(54) Title: DETECTION OF HLA-G

● 3C/G4-biotin + 3C/G4
○ 3C/G4-biotin + 2C/C8

(57) Abstract: The present invention relates to the detection of HLA-G. Antibodies to both soluble and membrane bound HLA-G are disclosed. Exemplary antibodies include 2C/C8, 3C/G4, and 4H84. 2C/C8 and 4H84 antibodies bind to the same region of HLA-G, which is a different region than that bound by 3C/G4. Methods of detection and diagnosis are disclosed as well as kits, including a miniaturized assay suitable for a clinical setting. Further, a method of selecting an embryo for in vitro fertilization is disclosed. A sandwich ELISA test is provide using two antibodies that bind to HLA-G at different regions. The HLA-G test according to the invention is over 1000 times more selective in binding to HLA-G than to antigens HLA-A_2, HLA-B_4, HLA-C, or mixed WBC preparations.

DETECTION OF HLA-G

FIELD OF INVENTION

The present invention relates to the detection of HLA-G. The present invention provides antigens for generating specific antibodies to both soluble and membrane bound HLA-G, as well as exemplary antibodies.

5   ## BACKGROUND OF THE INVENTION

A central question in pregnancy is how the fetal-placental unit avoids maternal immune rejection. Although fetal and maternal cells interact throughout pregnancy, the fetus typically remains a privileged site, not subject to rejection. It is likely that the particular nature of the cells at the fetal-maternal interface and their

10   products help prevent rejection of the fetus by the maternal immune system.

Implantation and placental development physically connect the mammalian embryo to the maternal uterus. Establishing this connection is essential for subsequent development. The initial developmental events which occur in the embryo set aside unique extraembryonic cellular lineages which are the precursors

15   of the placenta. The first differentiation event gives rise to trophoblasts, which are specialized epithelial cells of the placenta that physically connect the embryo and the uterus, see for example Cross *et al. (1994), Science 266:* 1508 for a review of the events surrounding implantation and formation of the placenta.

After fertilization in the oviduct, a series of cell divisions create a mass of

20   totipotent cells called the morula. The first differentiation event occurs after compaction of the morula, leading to formation of the blastocyst. Cells of the trophoblast lineage are formed based upon their position in the morula in a complex cascade of inter- and intra-cell signaling events. In primates, implantation of the blastocyst occurs shortly after the blastocyst hatches from the zona pellucida.

25   The uterus is made receptive to implantation as a result of events controlled largely by production of estrogen and progesterone from the ovaries. During implantation, trophoblasts attach to the receptive uterine epithelium initiating several changes in the endometrium. Vascular changes occur, such as increased permeability

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of uterine blood vessels, and inflammatory cells are recruited to the implantation site. Proinflammatory cytokines are produced in the uterus and several cellular changes occur. For example, the uterine epithelium is lost and decidual cells undergo an epithelioid transition and proliferate, producing a massively thickened uterine wall. The decidua also contains abundant macrophages, lymphocytes and other

5   bone-marrow derived cells with unusual properties such as reduced alloreactivity, and responsiveness to stimulation by CD3 antibody.

After implantation in humans, distinct populations of differentiated trophoblasts form. Proliferative cytotrophoblast stem cells are anchored to basement membranes surrounding a stromal core in two types of chorionic villi. In floating

10   villi, cytotrophoblast stem cells detach from the underlying basement membrane and fuse to form a syncytium, a polynucleate cell, which covers the villus and is in direct contact with maternal blood. In anchoring villi, cytotrophoblast stem cells differentiate, by detaching from their basement membrane and aggregating to form columns of mononuclear cells which attach to and invade the uterine decidua

15   (interstitial invasion) and its arterial system (endovascular invasion). Interstitial invasion puts cytotrophoblasts in direct contact with the highly specialized subset of leukocytes that are home to the uterus during pregnancy. Endovascular invasion puts cytotrophoblasts, like the syncytiotrophoblasts covering the anchoring villi, in direct contact with maternal blood. Thus, antigen presentation by trophoblasts at the

20   maternal-fetal interface is an important component of maternal immunological responses during pregnancy.

MHC class I molecules and the peptides they present regulate alloreactivity, see for example Sherman, at al. (1993), Annu. Rev. Immunol. 11: 385. Thus, one key to understanding maternal tolerance of the fetal semi-allograft lies in studying

25   trophoblast expression of class I molecules. The molecule HLA-G, which is expressed by placental cells, was cloned in a search for novel class I genes encoded by the human MHC, see for example Geraghty et al. (1987) Proc. Natl. Acad. Sci. U. S. A. 84: 9145. The gene has an intron/exon organization identical to that of the class Ia genes (HLA-A, -B and -C), and the HLA-G protein product has 86%

30   sequence identity to the class I consensus sequence, see for example Parham et al.

(1988) *Proc. Natl. Acad. Sci U. S. A.* 85: 4005. HLA-G has a lower molecular mass

(37-39 kDa) then class la molecules due to a stop codon in exon 6 that results in the

deletion of all but 6 amino acids in the cytoplasmic tail, see for example Shimizu *et*

al. (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85: 227. With regard to the 5' flanking

region of the gene, the HLA-G promoter has elements (e.g., AP-1, NFkB) similar to

5   sequences found in class la genes, but lacks an interferon response element,

suggesting novel transcriptional regulatory mechanisms. The primary HLA-G RNA

transcript is also differentially spliced; in addition to the full length mRNA,

transcripts are produced that lack either exon two, both exons two and three (see for

example Ishitani and Geraghty (1992) *Proc, Natl, Acad. Sci. U. S. A. 85:* 3947), or

10   exon four (see for example Kirszenbaum *et al.* (1994) *Proc. Natl. Acad. Sci; U. S. A.*

91:4209). To what extent these alternatively spliced mRNAs are translated is

unclear. A soluble form of HLA-G encoded by an mRNA containing intron 4 was

described by Fujii *et al.* (1994) *J. Immunol.* 153: 5516.

HLA-A, -B and -C are highly polymorphic, but HLA-G appears to exhibit

15   relatively less polymorphism. Immunoprecipitation of HLA-G from 13 individuals

and a human choriocarcinoma malignant trophoblast cell line showed identical

two-dimensional electrophoretic profiles, suggesting reduced polymorphism at this

locus. Genomic and cDNA sequence data also indicate that HLA-G has relatively

limited polymorphism. However, there is suggestion that at least in some

20   populations, i.e., African Americans, HLA-G exhibits substantial polymorphism, see

for example van der Ven and Ober (1994) *J. Immunol.* 153: 5628. Whether HLA-G

is complexed with endogenous trophoblast peptides and how this repertoire is

affected by its degree of polymorphism remains to be determined.

HLA-G is not generally expressed in non-pregnant adults, making it a

25   suitable marker for the diagnosis and monitoring of pregnancy, and for detecting

cytotrophoblasts from biological fluids. In addition, HLA-G levels in the maternal

blood are indicative of the vigor of cytotrophoblast invasion and the corresponding

health of the placental-maternal interface. Prior to the work of *Fisher et al.*, as

described in International Publication No. WO 96/31604, suitable antibodies to

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HLA-G had not been obtained, due to the high similarity of HLA-G to class Ia molecules which are expressed in adults.

One complication of pregnancy in which an abnormal maternal immune response to the fetus has been implicated is pre-eclampsia. This condition is characterized by development of the classic triad of hypertension, edema, and

5   proteinuria, usually in the third trimester of pregnancy, and is usually associated with signs of neurologic hyperirritability, which may eventually result in grand mal seizures. This pregnancy complication remains one of the major causes of maternal and perinatal mortality in both North American and the developing world, see for example Rochat RW, *et al.* (1988) Maternal mortality in the United States: report

10   from the Maternal Mortality Collaborative, *Obstet Gynecol*, 72(1):91-7, and AbouZahr C, *et al.* (1996) Maternal mortality, *World Health Stat Q*, 49(2):77-87.

Despite extensive research into pre-eclampsia, the underlying cause or causes remain unknown. Evidence accumulated thus far strongly suggests that the causative agent is the placenta, see for example Redman (1991) *Placenta*, 12(4):301-8.

15   Pre-eclampsia can occur with hydatidiform mole or choriocarcinoma, see for example Chun D, *et al.* (1964) *J. Obstet. Gynecol. Br. Commonw.* 71:180-184, where no fetus is present. The only cure for this widely occurring and life threatening disease is delivery of the placenta. Elucidating the exact role played by the placenta in this disease has been difficult because fundamental aspects of the

20   normal biology of this interesting organ are not well understood. The commonly associated finding of incomplete cytotrophoblast invasion of the spiral arteries suggests impairment of placentation, see for example Lim *et al.* (1997) *Am. J. Pathol.*, 151(6):1809 -18. This lack of trophoblast invasion in pre-eclampsia could be either a primary event or a secondary response to an abnormal immune reaction

. 25   to the fetus. Several features of this disease suggest that an abnormal response of the mother to the fetus may be the basic defect in this disease, see for example Redman (1991) *Semin Perinatol*, 15(3):257-62. Pre-eclampsia is most likely to occur in primagravidas, and is usually less severe or absent in subsequent pregnancies, but it may reoccur in the same mother if she changes sexual partners. This suggests that an

inappropriate response to some paternally derived antigen, possibly HLA-G, may result in pre-eclampsia.

HLA-G expression has been found to be decreased in the placental bed of women with pre-eclampsia, see for example Hara N, *et al.* (1996) *Am J. Reprod. Immunol.*, 36(6):349-58. However, there is currently no good predictive test for the

5   development of pre-eclampsia, even though shallow invasion in the first trimester sets up conditions for the clinical signs that manifest later in pregnancy, most commonly late in the third trimester.

Although International Publication No. WO 96/31604 (Fisher *et al.*) teaches the formation of anti-HLA-G monoclonal antibodies 1B8 and 3F6, for use in the

10   detection of HLA-G, there is no suggestion that these antibodies be used in the detection of pre-eclampsia. Both of these antibodies bind to a subsequence in the α1 domain of HLA-G. Because these antibodies detect the same region of HLA-G, there is no synergy to be derived from their combined use in detecting HLA-G. There is a need for an improved detection method for HLA-G in which more than

15   one antibody is used, and in which the antibodies used do not compete for the same binding region. Further, there is a need for an HLA-G detection method, such as an ELISA which has a high binding selectivity for HLA-G and low binding selectivity for HLA-A_2, HLA-B_4, HLA-C and WBC.

Conventional methods for diagnosing pre-eclampsia or for detecting antigens

20   in a biological sample require such a sample to be sent away to a laboratory for analysis, and do not allow rapid detection in a clinical setting.

SUMMARY OF INVENTION

It is object of the present intention to provide an improved test for the measurement of HLA-G in serum levels.

25   It is another object to provide antigens by generating specific antibodies to both soluble and membrane bound HLA-G.

It is yet a further object of the invention to provide specific antibodies to HLA-G.

It is still a further object of the invention to provide an improved diagnostic test for pre-eclampsia.

Another object of the invention is to provide a miniaturized detection assay, conducive to obtaining rapid results in a clinical setting.

A method is provided for the purification of naturally occurring of HLA-G

5   from human placenta. The method uses any one of the antibodies 3C/G4, 2C/C8, and 4H84.

The invention provides hybridoma 2C/C8, deposited with the International Depositary Authority of Canada (IDAC), Health Canada, 1015 Arlington St., Winnipeg, Manitoba, R3E 3R2 Canada as Accession Number: IDAC 130900-1,

10   deposited on September 13, 2000. This hybridoma produces 2C/C8 antibodies, and is deposited in accordance with all aspects of the Budapest Treaty.

The invention also provides hybridoma 3C/G4, also deposited with the International Depositary Authority of Canada (IDAC). Hybridoma 3C/G4 was afforded Accession Number: IDAC 130900-2, deposited on September 13, 2000.

15   This hybridoma produces 3C/G4 antibodies, and is deposited in accordance with all aspects of the Budapest Treaty.

It has been found that the monoclonal antibody 2C/C8 for HLA-G has substantially the same binding site an the monoclonal antibody 4H84.

The invention provides a method for detecting HLA-G in a biological sample

20   comprising the steps of: (a) depositing a biological sample on a support having an immobilized anti-HLA-G antibody bound thereto, wherein the immobilized anti-HLA-G antibody binds to a first region of HLA-G; (b) contacting the support having the biological sample deposited thereon with an HLA-G label, wherein the label binds to a second region of HLA-G; and (c) detecting the label. In an optional

25   embodiment of the invention, the immobilized anti-HLA-G antibody is 2C/C8, 3C/G4 or 4H84.

Further, the invention relates to a method for identifying an HLA-G indicative condition in a patient comprising the steps of obtaining a biological sample from the patient, followed by the above-noted method for detecting HLA-G.

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The invention also provides a method for determining potential for successful implantation of an embryo comprising the steps of obtaining a sample of a fluid medium incubating the embryo followed by the above-noted method for detecting HLA-G.

According to the invention, there is provided a kit comprising: (a) a support
5   having an immobilized anti-HLA-G antibody bound thereto; wherein the immobilized anti-HLA-G antibody binds to a first region of HLA-G; and (b) an HLA-G label which binding to a second region of HLA-G. According to an optional embodiment of the invention, the HLA-G label comprises a mobile anti-HLA-G antibody having a reporter molecule bound thereto. The reporter molecule may be
10   any molecule which is detectable in a quantitative or nearly quantitative manner. For example, a reporter molecule may be a colorimetric agent, a fluorometric agent, a radioisotope, or an enzymatic agents having a detectable end-point.

By the term "mobile" as it is used herein with respect to a mobile anti-HLA-G antibody, it is merely indicating that the antibody is not bound to the support,
15   unlike the immobilized antibody, which is bound to the support. Of course, the mobile antibody may be bound to other molecules, such as the reporter molecule. The mobile antibody may be provided in any manner known in ELISA technology, such as in a fluid solution.

When the HLA-G label is a mobile anti-HLA-G antibody having a reporter
20   molecule bound thereto, an optional embodiment of the invention is provided as follows. The immobilized anti-HLA-G antibody may be consisting of 2C/C8, 3C/G4 or 4H84. The mobile anti-HLA-G antibody is then either 2C/C8, 3C/G4, or 4H84, provided that the mobile and immobilized antibodies bind to different regions of HLA-G, referred to herein as a "first region" and a "second region". In this way,
25   there is no competitive binding between the mobile and immobilized antibodies. Since 2C/C8 and 4H84 bind to the same region of HLA-G, in this embodiment, either the immobilized anti-HLA-G antibody or the mobile anti-HLA-G antibody is 3C/G4. For example, if the immobilized anti-HLA-G antibody is 2C/C8 or 4H84, the mobile anti-HLA-G antibody is 3C/G4. Conversely, if the immobilized anti-

HLA-G antibody is 3C/G4, the mobile anti-HLA-G antibody is either 2C/C8 or 4H84.

The method according to the invention may optionally comprise the step of measuring HLA-G by comparing the quantity of label detected in the biological sample with an HLA-G standard. One possible source of the HLA-G standard is

5   that obtained from a human placenta.

The method according to the invention involves a biological sample. Such a sample may be selected from, but is not limited to amniotic fluid, a medium contacting an embryo, a tissue sample, a blood sample, a medium contacting a tissue sample, and a medium contacting a cell, for example when isolated cells are used.

10   The inventive method may be used to diagnose or detect an HLA-G indicative condition. In this embodiment, a control value for an HLA-G indicative condition can be compared with the quantity of HLA-G found in the sample. Certain conditions may be indicated if HLA-G is low or absent, while others may be indicated by increased levels of HLA-G. One of skill in the art could easily

15   determine the indicative levels useful in diagnosing a condition. Such HLA-G indicative conditions may include, but are not limited to pre-eclampsia, increased risk of pre-eclampsia, adverse fetal outcome, increased risk of adverse fetal outcome, cancer, or increased risk of cancer development.

Hybridomas 2C/C8 and 3C/G4, both deposited at the International

20   Depositary Authority of Canada on September 13, 2000 and having IDAC Accession Numbers IDAC 130900-1 and 130900-2 are encompassed by the invention. Antibodies produced by these hybridomas, ELISAs involving these antibodies also fall within the scope of the invention.

The method according to an embodiment of the invention may comprise a

25   miniaturized assay conducted on a chip. A chip may refer to a support formed of any plastic or inert polymer such as polystyrene, a cellulose or nitrocellulose support, a silicon-based or metal-based support, or any other platform as is known in the art. According to one embodiment of the invention, an immobilized anti-HLA-G antibody is bound to a polystyrene chip, sized to receive a sample volume ranging

30   from about 5 to 50 μl. A miniaturized assay can be conducted in a clinical setting,

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for example for determining HLA-G in a sample of embryo culture medium, for
predicting successful embryo implantation. Larger or smaller sample volume assays
are also within the scope of the invention, but this embodiment is conducive to an
on-site test which does not require a sample to be sent away for laboratory analysis.
Use of such a chip, for example within a kit would be advantageous for determining

5   potential for successful implantation of an embryo in *in vitro* fertility procedures.

The invention further provides a method for selecting an embryo for *in vitro*
fertilization (IVF) comprising the steps of detecting soluble HLA-G secreted by an
embryo into an incubation medium, and choosing an embryo secreting soluble HLA-
G at a minimum level of 0.01 µg/ml of incubation medium. Optionally, this method

10   may be combined with the evaluation of embryo cleavage rate, and based on both
parameters, an embryo is chosen for IVF. In this method for selecting an embryo,
the step of detecting soluble HLA-G may be conducted according to the method for
detecting HLA-G according to the invention.

Unless defined otherwise, all terms used herein have the same meaning as

15.   commonly understood by one of ordinary skill in the technical art to which this
invention belongs. Although methods and materials similar or equivalent to those
described herein can be used in the practice or testing of the invention, the preferred
methods and materials are described.

The term "antibody" refers to a polypeptide substantially encoded by an

20   immunoglobulin gene or immunoglobulin genes, or fragments thereof. The
recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta,
epsilon and mu constant region genes, as well as myriad immunoglobulin variable
region genes. Light chains are classified as either kappa or lambda. Heavy chains are
classified as gamma, mu, alpha, delta, or epsilon, which in turn define the

25   immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

An exemplary immunoglobulin, antibody, structural unit comprises a
tetramer. Each tetramer is composed of two identical pairs of polypeptide chains,
each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD).
The N-terminus of each chain defines a variable region of about, for example, 100 or

30   more amino acids primarily responsible for antigen recognition. The terms variable

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light chain (V_L) and variable heavy chain (V_H) refer to these light and heavy chains respectively.

Antibodies can exist, for example, as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Pepsin digests an antibody below the disulfide linkages in the hinge region to

5   produce $F(ab)'_2$, a dimer of Fab which itself is a light chain joined to V_H-C_H1 by a disulfide bond. The $F(ab)'_2$ may be reduced under mild conditions to break the disulfide linkage in the hinge region thereby converting the $F(ab)'_2$ dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region broken. While various antibody fragments are defined in terms of the digestion of an

10   intact antibody, one of skill will appreciate that such Fab' fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies or those synthesized *de novo* using recombinant DNA methodologies.

15   The term "biological sample or fluid" refers to material derived from a living organism, including but not limited to blood and blood derivatives, cervicovaginal secretions, amniotic fluid, cord blood, urine, tissues, bones and cells.

The term "blood sample" as used herein includes whole blood or derivatives of whole blood well known to those of skill in the art. Thus a blood sample includes

20   the various fractionated forms of blood such as plasma or serum and whole or fractionated blood which additionally comprises various diluents as may be added to facilitate storage or processing in a particular assay. Such diluents are well known to those of skill in the art and include various buffers, anticoagulants, preservatives and the like.

25   The term "HLA-G" refers to human leukocyte antigen G and unless otherwise stated includes both the soluble and insoluble forms. The term may in appropriate context refer to either the antigen or the genetic locus.

The term "immunoassay" is an analysis or methodology that utilizes an antibody to specifically bind an analyte. The immunoassay is characterized by the

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use of specific binding properties of at least one particular antibody to isolate, target, or quantify the analyte.

The terms "isolated", "purified", or "biologically pure" refer to material which is substantially or essentially free from components which normally accompany it as found in its native state.

5   The term "nucleic acid" refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues of natural nucleotide that can function in a similar manner as naturally occurring nucleotides, for example, incorporating conservative substitutions as is well known in the art.

10   The term "nucleic acid probe" refers to a molecule which binds to a specific sequence or subsequence of a nucleic acid. A probe is preferably a nucleic acid which binds through complementary base pairing to the full sequence or to a subsequence of a target nucleic acid. It will be understood by one of skill in the art that probes may bind target sequences lacking complete complementarity with the

15   probe sequence depending upon the stringency of the hybridization conditions. The probes are preferably directly labeled as with isotopes, chromophores, lumiphores, chromogens, or indirectly labeled such as with biotin to which a streptavidin complex may later bind. By assaying for the presence or absence of the probe, one can detect the presence or absence of the select sequence or subsequence.

20   The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residues is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

25   A "label" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include ^{32}P, fluorescent dyes, electron-dense reagents, colorimetric, enzymes, for example, as commonly used in ELISA, biotin, dioxigenin, or haptens and proteins for which antisera or monoclonal antibodies are available can be made detectable.

A "labeled nucleic acid probe" is a nucleic acid probe that is bound, either covalently, through a linker, or through ionic, van der Waals or hydrogen bonds to a label such that the presence of the probe may be detected by detecting the presence of the label bound to the probe.

The term "recombinant" when used with reference to a cell indicates that the

5   cell contains nucleic acid with an origin exogenous to the cell. Thus, for example, recombinant cells replicate and/or express genes that are not found within the native (non-recombinant) form of the cell.

The term "identical" in the context of two nucleic acid or polypeptide sequences refers to the residues in the two sequences which are the same when

10   aligned for maximum correspondence.

The phrases "specifically binds to", "specifically hybridizes to" or "specifically immunoreactive with", when referring to an antibody indicate a binding reaction which is determinative of the presence of the protein in a heterogeneous population of proteins and other biological substances. Thus, under designated

15   immunoassay conditions, the specified antibodies bind preferentially to a particular protein and do not bind in a significant amount to other proteins present in a biological sample or fluid. Specific binding to a protein under such conditions requires an antibody that is selected for its specificity of binding with a particular protein.

20   Herein, a particular antibody and the cell which produces the antibody may be referred to by the same term, however, one of skill in the art can easily distinguish whether it is the antibody or the cell to which any particular instance pertains. Thus, a hybridoma cell line may be referred to by the name of its defining antibody.

25   **BRIEF DESCRIPTION OF THE DRAWINGS**

The invention will be described in more detail in the following way by way of example only and with reference to the attached drawings wherein:

Figure 1 shows binding affinities of 2C/C8 (o) and 3C/G4 (•) antibodies to HLA-G.

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Figure 2 shows an antibody competition assay for 3C/G4 and 2C/C8.

Figure 3A shows western blotting of some ELISA antibodies, 4H84 and 3C/G4, for HLA-G.

Figure 3B shows western blotting of some ELISA antibodies, 2C/C8 and 3C/G4, for HLA-G.

5   Figure 4 shows separation of an antibody-HRP conjugate from uncoupled HRP by P- 100 gel filtration chromatography.

Figure 5 shows immune and enzyme activity of an antibody-HRP conjugate.

Figure 6 shows a comparison of standard curves between a direct and an indirect coat antibody.

10   Figure 7 shows determination of concentrations of streptoavidin and 3C/G4-biotin conjugate for an indirect coat antibody.

Figure 8 shows purified HLA-G protein characterized by SDS-PAGE™ and Western blot.

Figure 9 shows purification of HLA-G protein, checked by ELISA. Various

15   concentrations of purified HLA-G protein were first coated and then incubated with 3C/G4 antibody plus anti-mouse antibody (o) or with an anti-mouse antibody alone (●).

Figure 10 shows a standard curve and a parallelism test of HLA-G in serum.

Figure 11 illustrates a typical standard curve determined using the HLA-G

20   ELISA according to the invention.

Figure 12 shows a correlation between HLA-G protein concentrations in placenta and serum at term. HLA-G concentrations in normal pregnant women at term represented by the closed circles (●) and the pre-eclampsia by open circles (o).

Figure 13 shows serum HLA-G levels during the first trimester of pregnancy

25   in normal women (●) and in pre-eclamptic women (o).

Figure 14 illustrates serum HLA-G levels during the second trimester of pregnancy in normal women (●) and in pre-eclamptic women (o).

Figure 15 shows serum HLA-G levels during the third trimester of pregnancy in normal women (●) and in pre-eclamptic women (o).

Figure 16 illustrates an antibody competition assay to compare antibody binding sites of two HLA-G monoclonal antibodies, 4H84 (●) and 3C/G4 (o).

Figure 17 shows a comparison of the number of blastomeres in samples testing negative versus those testing positive for HLA-G in medium.

Figure 18 illustrates pregnancy rates of (A) HLA-G positive and negative

5   groups and (B) groups having average number of blastomeres larger than 6 and less than or equal to 6.

Figure 19 shows pregnancy rates compared among various groups combining sHLA-G detection and embryo cleavage rate.

DETAILED DESCRIPTION OF THE INVENTION

10   An ELISA has been developed for measurement of serum levels of HLA-G. Using this ELISA, it has been found that women diagnosed with pre-eclampsia had significantly lower levels of HLA-G than normal controls. In addition, it has been found that levels of HLA-G per mg of placental protein were lower in these patients and there was a linear correlation between HLA-G levels in serum and placental

15   tissue of the same woman. This finding leads the way to test women earlier in pregnancy for the presence of HLA-G in their serum. Testing women early in pregnancy for the presence of HLA-G in their serum provides a screening/predictive test for pre-eclampsia.

A critical period of fetal development for survival is that of the early pre-

20   implantation embryo and therefore determining whether HLA-G is expressed during this period is important for understanding its possible role as an embryo protectant. Jurisicova A., *et al. (Fertil. Steril.* (1996) 65(5):997-1002) reported that it is possible to detect HLA-G heavy chain mRNA in 40% of blastocysts, in some embryos at earlier pre-blastocyst cleavage stages of development (2-4 cell, 5-8 cell,

25   and *morula)* and in some unfertilized oocytes. In concordance with mRNA data, a similar proportion of embryos stained positive for HLA-G immunohistochemistry (Jurisicova, A., *et al.* (1996) *Proc. Natl. Acad. Sci. USA.* 93:161-165). In addition, it was also found that patients who became pregnant and did not have a fetal loss, had a significantly higher proportion of HLA-G positive sibling blastocysts than patients

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who did not conceive. These studies represented the first report demonstrating the

presence of protein and mRNA for the heavy chain of HLA-G, a non-classical class I

MHC antigen, and for β2m throughout the whole course of human pre-implantation

development from the oocyte to blastocyst stages.

Currently, *in vitro* fertility (IVF) laboratories are able to select pre-embryos

5   only on the basis of their morphology and rate of *in vitro* cleavage during the first 48

to 72 hours after fertilization. These criteria are useful, but not always good

indicators of developmental potential. In most cases, 3 or 4 embryos are chosen

based on these relatively crude indicators and then transferred into the uterine cavity.

If additional, more stringent pre-embryo selection criteria were available, based on

10   biochemical, genetic or developmental parameters, it would be possible to transfer

one or two healthy pre-embryos, which have the highest chance of survival, without

exposing patients to the psychological trauma caused by recurrent embryo

implantation failure, spontaneous abortions, multiple IVF trials or the risk of

multiple pregnancy. Therefore, a more predictive test for successful implantation

15   would be invaluable.

In addition to the aforementioned utility for a selective HLA-G test, there are

other possible utilities. A determination of HLA-G levels in serum is also believed

to be indicative of cancer. Cancer cells mask themselves by producing protein

indicative of normal cells. In this regard, a selective HLA-G test would be desirable.

20   ***Preparation of Monoclonal Antibodies Against HLA-G.*** Two hybridoma

cell lines named 2C/C8 and 3C/G4 that produce monoclonal antibodies against

HLA-G have been made. The hybridomas are deposited with IDAC as Accession

Numbers IDAC 130900-1 and 130900-2, respectively. The cell lines were obtained

by using purified HLA-G protein to immunize three week old female BALA/c mice

25   according to the procedure described by Harlow Ed and lane D: *Antibodies, a*

Laboratory Manual, Cold Spring Laboratory; NY, 1988, which reference is

incorporated herein in its entirety. By using a mouse antibody typing kit

(Amersham™) the subtype of monoclonal antibody produced by 3C/G4 cell line is

determined to be IgG_3 while monoclonal antibody secreted by 2C/C8 cell line is

30   determined to be Ig A.

Figure 1 illustrates binding affinity of 2C/C8 (O) and 3C/G4 (●) antibodies to HLA-G. The 3C/G4 antibody has a higher affinity than 2C/C8 antibody, so that advantageously, 3C/G4 may be used as a first primary antibody and 2C/C8 may be used as a second primary antibody in a sandwich ELISA system in accordance with an embodiment of the present invention.

5   To obtain the antibodies, the cell lines are cultured in a hybridoma culture medium constructed with 410 ml Optimen™ medium (Gibco™), 5 ml TANGOO™ (Sigma™), 0.5 ml ITS (Sigma) and 75 ml FBS (Sigma) for 7 to 10 days until all hybridoma cells die. The debris is removed by centrifugation at 400 g for 10 minutes and supernatant is pooled. The purification of the antibodies is carried out by using a

10   protein G column (Sigma) according to the procedure described by Yie S.M., in *Purification of HLA-G, a Laboratory Manual*; 1997, which reference is incorporated herein in its entirety. The purified antibodies were then concentrated by using a molecular weight 10,000 cut-off filter (Millipore™) and centrifugation at 3000 rpm for 30 minutes at 4 °C. Antibodies were stored at -80 °C until use. Antibody yields

15   were approximately 10 µg/ml supernatant.

Figure 2 illustrates an antibody competition assay for 3C/G4 and 2C/C8. By using a competition antibody capture assay it is demonstrated that the two antibodies bind different binding epitopes of HLA-G molecule.

Specific antibodies were measured by three different approaches, i.e.,

20   immunocytochemistry, western blot and ELISA. The antibodies can specifically stain cytotrophoblasts isolated from human first trimester placenta while a negative stain was found in human white blood cell slides, see for example McMaster M *et al.* (1995) *J. Immunology* 154:3 771.

Figure 3A shows western blotting of some ELISA antibodies, 4H/84 and

25   3C/G4, for HLA-G. Results are shown in lane 1 for JEG-3, lane 2 shows WBC, lane 3 shows LCL, and lane 4 shows C1R.

Figure 3B shows western blotting of some ELISA antibodies, 2C/C8 and 3C/G4, for HLA-G. Results are shown in lane 1 for HLA-G, lane 2 shows WBC, lane 3 shows JEG, and lane 4 shows C1R-Gs. Western blotting clearly shows that

30   the antibodies only blot HLA-G.

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Table 1 shows that there is no cross reaction of the inventive antibodies with other classical HLA class I antigens, as determined by antigen capture ELISA. These data further confirm the specificity of the inventive antibodies for HLA-G.

Table 1

Cross Reactions of 3C/G4 and 2C/C8 Antibodies

Compounds	Cross-activity %
HLA-G	100
HLA-A_2	0.01
HLA-B_4	0.01
HLA-C	0.001
Mixed WBC	0.005

Labeling Biotin to 3C/G4 Antibody. For technical directions for a general procedure the reader is directed to Wilchek M., *et al.* (1988) *Anal Biochem* 171:1, which reference is incorporated herein in its entirety.

The purified antibody (2 mg/ml) was extensively dialyzed against 0.1 M PBS/0.15 M NaCl (saline) buffer (pH = 7.2) for 24 hours at 4 °C. Buffer was changed at least three times during the dialysis. 1 mg of biotin (Sigma™) was dissolved in 30 μl of DMSO (Sigma™) and 70 μl of 0.1 M PBS/saline buffer was then added. The antibody solution was immediately mixed with 38 μl of biotin solution on an end-over-end rocker for 2 hours at room temperature. Unlabeled biotin was separated by using a Sephadex™-25 column (P-10 column, Pharmarcia™) and eluted by 0.01 M PBS buffer (pH = 7.2), at a volume of 1 ml per fraction. The presence of antibody-biotin conjugate in each fraction was checked by using Bio-Rad™ protein assay kit. The fractions with high concentration of antibody-biotin conjugate were pooled. Aliquots of the conjugate was stored at -80 °C until use.

Table 2 provides buffer formulations used in the labeling procedure.

Table 2

Buffer Formulations

0.1 M PBS/saline	68 ml of 1 M Na_2HPO_4, 32 ml of 1 M Na_2HPO_4, and 8.8 g NaCl. Add ddH_2O to 1 L
0.01 M PBS	6.8 ml of 1 M Na_2HPO_4, and 3.9 ml of 1 M Na_2HPO_4. Add ddH_2O to 1 L

5   *Labeling Horse-Radish Peroxidase (HRP) to 2C/C8 Antibody.* For

technical directions for a general procedure the reader is directed to Hashida S., *et al.*

(1984) *J Applied Biochemistry* 6:56, which reference is incorporated herein in its

entirety. To digest antibody to $F(ab)_2$, the following procedure was followed. 3 mg

of antibody purified by G-protein chromatography and eluted with 0.1 M glycine-

10   HCl buffer (pH = 3.0) was digested with 60 µg of pepsin (Sigma™) at 37 °C for 16

hours. The enzyme activity was stopped by adjusting pH to 8.0 with 1 N NaOH. A

P-100 (Bio-Rad™) gel filtration column (1 x 50 cm) was used to purify the digested

$F(ab)_2$ eluted with 0.1 M PBS buffer (pH = 6.0). Fractions with $F(ab)_2$ (MW = 4,600)

were pooled and concentrated using filter centrifugation.

15   The introduction of a thiol group to $F(ab)_2$ was conducted as follows. 1 ml

$F(ab)_2$ solution was mixed with 0.1 ml of 1 M 2-mercaptoethylamine (Sigma™) in

0.1 M PBS with 5 mM EDTA and incubated at 37 °C for 2 hours. The mixture was

purified on a P-10 column, eluted with 0.1 M PBS (pH = 6.0) and concentrated by

filter centrifugation.

20   To introduce a maleimide group to HRP, the following procedure was used.

5 mg HRP was dissolved in 0.1 M PBS/0.1 M NaCl/0.1% BSA. Subsequently, 2.6

mg of SSC was dissolved in 0.075 ml NN-dimethylformamide. The HRP solution

was incubated with SSC solution at 4 °C overnight. The HRP-maleimide was

purified on a P-10 column and concentrated by filter centrifugation.

25   Conjugation of HRP to $F(ab)_2$ was conducted as follows. 1 mg of HRP-

maleimide was incubated with 1.2 mg of thiol $F(ab)_2$ in 0.1 M PBS (pH = 6.0) with

18

5 mM EDTA at 4 °C for 20 hours. The resulting F(ab)$_2$-HRP conjugation was purified on P-100 column.

Figure 4 shows a purification profile of the F(ab)$_2$-HRP conjugate, illustrating fractional separation of an antibody-HRP conjugate from uncoupled HRP by P-100 gel filtration chromatography.

5   Figure 5 illustrates the immunological and enzyme activity of the antibody-HRP conjugate obtained in the purification shown in Figure 4. 100 µl/well of placenta cell lysate was first coated on an ELISA plate. Various concentrations of Ab-HRP conjugate were incubated at 4 °C overnight. Enzyme activity was determined by o-phenylenedianine (OPD) color reaction at 490 nm. The method of

10   assaying a bound HLA-G that is labeled may be selected from many different forms of detection, for example it is possible to assess bound HLA-G by detection with a standard streptavidin-HRP/o-phenylenediamine substrate colour reaction in an ELISA plate reader.

Coating Microplates. In this assay system an indirect coating method was

15   used. A comparison of assay sensitivity between direct and indirect coating antibody showed that indirect coating increased assay sensitivity.

Figure 6 shows a comparison of standard curves between direct coating and indirect coating of antibody. Direct coating is shown with a closed circle (●), and indirect coating is shown with an open circle (○). For direct coating, Y = 0.243 +

20   0.226X, r = 0.943; for indirect coating, Y = 0.297 + 0.61X, r = 0.984. A 96 well ELISA microplate (Nuc) with high binding capacity was selected. 100 µl of 2 µg/ml to streptoavidin (Sigma™) in 0.1 M Na$_2$HCO$_3$ buffer (pH = 8.0) was first coated on each well at 4 °C overnight. The plate was washed three times with a washing solution. The plate was blocked with 1% BSA in 0.1 M Na$_2$HCO$_3$ buffer at room

25   temperature for 3 to 4 hours. The block solution was decanted and dried. 100 µl of 3C/G4-biotin conjugate (5µg/ml) was added to each well and incubated at 4 °C overnight. The plate was washed again with washing solution. The plate was blocked again with 1% BSA in the Na$_2$HCO$_3$ buffer at room temperature for 3 to 4

hours. The block solution was decanted and dried. Coated plates were effective for
at least one month when stored at 4 °C.

Figure 7 shows a determination of concentrations of streptoavidin and
3C/G4-biotin conjugate for indirect coating antibody at concentrations ranging from
2 to 20 μg/ml. These data illustrate that it is possible for 2 μg/ml of streptoavidin
5   and 5 μg/ml 3C/G4-biotin conjugate to produce maximum binding.

Table 3 provides composition information for various solutions used herein
for coating microplates used in the ELISA according to the invention.

Table 3
Composition of Solutions Used in ELISA Preparation

0.1 M Na$_2$HCO$_3$	8.4 g Na$_2$HCO$_3$; 8.8g NaCl; 0.1g NaS. Adjust pH to 8.0 with NaC0$_3$; add ddH$_2$O to 1 L
Washing Solution	6.8 ml of 1 M Na$_2$HPO$_4$; 3.2 ml of 1 M NaH$_2$PO$_4$; 1 ml of Tween™-20. Add ddH$_2$O to 1 L
Block Solution	1 g of BSA powder Add 0.1 M Na$_2$HCO$_3$ buffer to 100 ml

Preparing HLA-G Standard. Purified HLA-G from human first trimester
placenta tissue was used as standard of this assay system. The purification of HLA-
15   G protein has been described in *Purification of HLA-G, a Laboratory Manual*, (Yie
S.M., 1997).

Figure 8 shows a purified placental HLA-G protein characterized by SDS-
PAGE™ and Western blot. Purity of the protein was over 95%. Lane A shows the
molecular weight maker; Lane B shows a fraction eluted from 4H84 mAb and
20   βMM1 mAb affinity columns; and Lane C shows immunoblot of purified HLA-G
protein using 4H84 mAb, illustrating locations of HLA-G and HLA-Gs.

Figure 9 shows a purification of HLA-G protein, as checked by ELISA.
Various concentrations of purified HLA-G protein were first coated and then
incubated with 3C/G4 antibody plus anti-mouse antibody as shown with open circles

20

(O), or with anti-mouse antibody alone as shown with closed circles (●). These results illustrate that no antibody contamination was detected.

Preparation of ELISA. 50 μl per well of HLA-G standard from 0 to 0.5 μg/ml prepared in 0.01M PBS and 50 μl of serum were added to plate and incubated at 4 °C overnight. The plate was washed three times with the washing solution. 50

5   μl per well of 2C/C8-HRP conjugate (10 μg/ml) in 0.01M PBS was added and incubated at room temperature for 1 to 2 hours. The plate was washed three times with the washing solution.

o-phenylenedianine (OPD) solution (1.5 mg/ml) (Sigma™) was prepared in 0.1 M ·acetate buffer (pH = 4.0). Hydrogen peroxide (4 μl of a 30% solution) was added to

10   10 ml OPD solution before adding 100 μl/well of substrate solution to wells. The plate was incubated at room temperature for 10 to 15 min. The reaction was stopped by adding 100 μl per well of 1N H_2SO_4. The plate was read at 490 nm in an ELISA reader.

Table 4 provides composition information for various solutions used in the

15   preparation of the ELISA plate.

Table 4

Composition for Various Solutions

0.1 M PBS/saline buffer pH = 7.2	6. 8 ml of 1 M Na_2HPO_4; 3.2 ml of 1 M NaH_2PO_4; 8.8 g NaCl. Add ddH_2O to 1L.
0.1 M Acetate buffer	4.4 g sodium acetate. Adjust pH to 4.5 with acetic acid. Add ddH_2O to 500 ml
OPD solution	15 mg OPD. Dissolved in 10 ml acetate buffer. Add 0.4 μl/ml of H_2O_2 to the solution just before adding to plate.

Performance Characteristics

Figure 10 illustrates a standard curve and parallelism test of HLA-G in serum. Serum values are shown with open circles (O), while the standard curve

25   values are shown with closed circles (●). The serum dilution curve takes the form Y

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= 0.121 + 0.106(logX), r = 0.992. The standard curve takes the form Y = 0.236 +

0.086(logX), r = 0.975. These results show that the binding in serum was identical

from HLA-G standard.

The sensitivity of the assay or lower limit of detection (LLD) was

determined by assaying replicates of zero and the standard curve. The mean signal of

5   zero plus 2 SD read in dose from the standard is the LLD $\cong$ 10 ng/ml. This ELISA is

specific for the measurement of natural HLA-G. It does not cross react with classical

class I antigens, as shown by results herein (see Table 1). The within and between

assay CV (variation) were 9.8 % and 11 %, respectively.

Table 5 shows mean assay recovery. The accuracy of the assay was estimated

10   by a recovery test of spiked purified HLA-G protein.

Table 5			
Assay Recovery			
HLA-G added	Measured Value	Calculated Value	Recovery
µg/ml	µg/ml	µg/ml	%
0	1.011	1.011	-
0.2	1.222	1.211	100.9
0.4	1.366	1.411	94.6
0.8	1.949	1.811	107.6

Miniaturization of the HLA-G Assay. Pregnancy rates from transferred

20   embryos having positive HLA-G secretion were significantly higher than those with

undetectable HLA-G. Thus, to ascertain whether an embryo to be transferred has an

increased likelihood of leading to pregnancy, a HLA-G detection assay can be

conducted. However, the time required for detection is extremely limited, for

example during an IVF procedure, and an accurate measurement taken within a

25   clinical setting is desirable. A miniaturized HLA-G detection method can be used in

a clinical setting so as to provide a rapid quantitative measurement of HLA-G in the

medium in which an embryo is incubated, without requiring that a sample be sent off for laboratory analysis. Such a rapid detection assay advantageously involves either a visually distinguishable end-point (ie-colorimetric) or may involve a reader which is either hand-held, or bench-top sized, using an enzymatic, fluorometric or colorimetric end-point.

5   A miniaturized assay according to the invention may involve any device or method for analyzing a biological sample, such as is known in the art. The assay may be present on a support, such as on a strip or a chip, formed of plastic, cellulose, or any other acceptable material. By conducting the assay according to the invention in a miniaturized fashion, there is a reduced requirement for larger pieces of

10   laboratory equipment. Further, there is less waste, as individual samples can be assessed with small amounts of biological fluids ranging from levels as low as 2 to 50 µl. A miniaturized assay, requiring a small sample volume allows detection even when sample size is severely reduced.

A miniaturized assay may have any acceptable form of detectable end-point

15   so that the quantity of HLA-G present may be readily determined.

Exemplary embodiments of the invention are discussed below, which are not to be considered limiting to the invention.

Example 1

ELISA for Detection of HLA-G in Serum

20   Serum samples were collected from non-pregnant healthy women, pregnant women at first trimester and term, patients after miscarriage, pre-eclampsia, and inter uterine growth retardation (IUGR). An ELISA assay according to the invention was conducted, using 96-well plates prepared with immobilized 3C/G4 antibody, in which samples were incubated. HRP-labeled 2C/C8 binding was

25   detected with OPD.

Table 6 illustrates mean values of serum HLA-G, as determined according to the invention. These data illustrate that the inventive assay is effective in the detection of serum HLA-G.

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Table 6

Serum HLA-G Values

Group	N	Mean ± SE (μg/ml)
Normal women	9	0.117 ± 0.008
Pregnant (first trimester)	9	0.898 ± 0.024
Pregnant (term)	14	0.896 ± 0.039
Miscarriage	9	0.355 ± 0.010
Pre-eclampsia	14	0.242 ± 0.021
IUGR	11	0.121 ± 0.005

Example 2

Serum and Placental HLA-G is Decreased in Women with Pre-Eclampsia

HLA-G levels in serum and placenta tissues from women with pre-eclampsia at term were compared with levels in normal pregnant women. HLA-G levels were determined by using an ELISA according to the invention in 20 subjects with pre-eclampsia and 14 control pregnant women. Both serum and placental HLA-G levels were significantly decreased in the pre-eclampsia group (median 0.026 μg/ml in serum and median 0.026 μg/mg protein in placenta), in comparison with normal pregnant women (median 0.093 μg/ml in serum and median 0.088 μg/mg protein in placenta, $p=0.0112$ and $p=0.0406$, respectively). There was a significant correlation between serum and placental HLA-G levels ($r=0.603$, $p=0.002$). The impaired expression of HLA-G suggested that there was an alternation of the maternal-fetal immune relationship in pre-eclampsia and decreased HLA-G plays a role in etiology of the disorder.

Pre-eclampsia is a disease that affects of approximately 7% of pregnant women. It remains the most leading cause of maternal death in many western countries. The illness is ultimately cured when the uterus is emptied of placenta tissues, implying a direct role of the placenta in it's pathophysiology. Despite extensive study, its underlying etiology still remains an enigma.

Many hypotheses have been proposed to elucidate the mechanisms of the development of pre-eclampsia. One implies that pre-eclampsia involves some type of incompatibility between mother's immune system and the semi-allogeneic fetal allograft. Some evidence has emerged to support the idea that the maternal immune system is activated in pre-eclamptic patients (Hara *et al.*, *Am. J. Reprod. Immunol*,

5   1995: 34:44-51). Expression of HLA-G protein, semi-quantitatively determined by immunohistochemistry and HLA-G mRNA northern blot analysis, is decreased in pre-eclamptic cytotrophoblasts compared with controls.

HLA-G is a non-classical class I antigen that is only expressed on extravillous cytophoblast of placenta and is almost monomorphic. It is also

10   secreted as a truncated form of protein. HLA-G may play a critical role in maternal immune tolerance to the fetus. A line of experimental evidences demonstrated that HLA-G may protect fetal-placental unit from natural killer cell lysis, cytotoxic T cell activity and modification of the type of maternal immune response.

If pre-eclampsia is due to deficient maternal immune accommodation to the fetus,

15   alternation of expression of HLA-G on pre-eclamptic placenta should be associated with etiology of the disease. Therefore, it is important to determine expression of HLA-G in pre-eclampsia by a quantitative method. A specific and sensitive sandwich ELISA was recently developed in our laboratory. By using this technique, HLA-G levels were measured in both serum and placenta tissue lysates from pre-

20   eclamptic patients to determine whether there is a significant alternation of HLA-G levels in both circulation and placenta in pre-eclamptic patients compared with normal pregnant women.

Materials and Methods. Twenty pre-eclamptic patients and 14 normal pregnant women were involved in this study. All the subjects were seen in the labor

25   and delivery suite of the Women's College Hospital (WCH), University of Toronto, Toronto, Canada from 1995 to 1997. This study was approved by the ethics committee of WHC. Pre-eclampsia was diagnosed according to the criteria of Chesley (*Obstet Gynecol*: 1985:65:423-425). None of these patients had a history of

25

intra uterine infections, pre-term labor or premature rupture of membrane.

Obstetrical summaries of the women with pre-eclampsia are shown in Table 7.

Table 7

Clinical Characteristics of Pre-eclamptic Patients

Criteria	Pre-eclampsia
Age (yrs)	25-40
Parity (times)	0
Blood Pressure (mmHg)	140-210/90-120
Proteinuria (dipstick)	2+-4+
Gestation Age (week)	29-40

10   Placenta tissues and serum samples were collected as follows. Blood taken

from patients and normal controls during labor was collected in serum separation

Vacutainer™ tubes (Becton Dickinson, NJ, USA). Serum was collected after

centrifugation for 10 minutes at 600g, aliquoted and stored at -70°C. Placental

tissues from patients and normal controls were obtained soon after the delivery and a

15   portion from the maternal surface was taken and stored in pieces at -70°C until

assay. Approximately 1g of the placental tissue was homogenized and lysed in Tris-

HCl lysate buffer containing 0.5% NP-40, 2.5 mM EDTA, and 200 mM phenyl-

methyl sulfonylfluoride (PMSF).

HLA-G protein was purified from first trimester placenta tissue, as described

20   in part by Deniz G et al., J Immunol 1994: 152:4255-4261. Briefly, first trimester

placentas were collected from patients who underwent elective terminations at the

Women's College Hospital, Toronto, Canada and stored at -80 °C until used.

Placenta tissue was homogenized in 50 mM Tris-HC1/0.15 M NaCl (Tris/NaCl)(pH

7.4) with a Brinkmann polytron homogenizer at 4 °C. The homogenized tissues

25   were washed three times with 0.05 M Tris-HCl/saline pH=7.4, and was resuspended

in 10 times the pellet volume of lysate buffer containing 50 mM Tris-HCl/0.15 M

NaCl, 0.5% Nonidet P-40 (NP-40), 2.5 mM ethylenediaminetetraacetic acid and

200 mM phenyl-methyl sulphonyl fluoride (PMSF) (Sigma). The suspension was

mixed gently on a rocker at 4 °C overnight. The suspension was centrifuged at 39

000 g for 30 minutes at 4 °C and the supernatant lysate was collected. The lysate

was obtained by centrifugation at 39,000 g for 30 minutes at 4°C. The placental

5   lysate was first passed over a Sepharose™ precolumn to eliminate non-specific

binding, and then applied to the following affinity column. HLA-G proteins were

then isolated by sequential 4H84 mAb (against HLA-G) and βMM1 mAb (against

β2-microglobumin) affinity columns.

After washing with 100 column volumes of 0.01 M phosphate-buffered

10 saline (PBS), pH 7.2, bound HLA-G was eluted with 0.1 M glycine-HCl buffer, pH

3.0. Fractions were immediately neutralized with 1 M Tris, pH 8.0. Using a

Bio-Rad protein assay kit (BioRad, Hercules, CA), fractions with high protein

concentrations were pooled. For additional purity, the pool was applied to the anti-

2m antibody, BMM 1, affinity column and HLA-G was eluted and neutralized as

15 above. Purification and characterization of isolated HLA were analyzed with

Western blot (McMaster et al, *J Immunol.* 1998:160:5922-5928) with the 4H84

mAb and Coomasie blue staining of the gel (Laemmli, Nature 1970: 227:680-685).

Immunoblotting involved purification of HLA-G and HLA-B7 proteins (1

g/lane) were resolved in 10% sodium dodecyl sulphate-polyacrylamide gel

20 electrophoresis (SDS-PAGE) gels and electroblotted onto nitrocellulose membranes

(Schleicher and Schuell, Keene, NH) (Towbin *et al.* (1979) *Proc Natl Acad Sci USA*

1979; 76:4350). Membranes were blocked in PBS containing 0.05% Tween-20

(Sigma, St. Louis, MO) and 5% Carnation™ non-fat dry milk (T-blotto) overnight at

4 °C , and then incubated with mAb 4H84, HC10 (hybridoma supernatant diluted

25 1:1 in T-blotto), or normal mouse serum (diluted 1:100 in T-blotto), for 1 hour at

room temperature. Membranes were washed three times (10 minutes/wash) with

PBS-Tween, and incubated with horseradish peroxidase-conjugated goat anti-mouse

immunoglobulin G (IgG-HRP; Amersham Life Science, Canada), diluted 1:2000 in

PBS-Tween, for 1 hour at room temperature. Membranes were washed three times

for 10 minutes each time and processed for chemiluminescence according to the manufacturer's instructions (ECL; Amersham Life Science) and exposed to autoradiography film (Kodak, Canada).

Two monoclonal antibodies against HLA-G protein were used for development of the ELISA used herein. The first was the mAb 4H84 which was a

5   gift of S. Fisher and M. McMaster (University of California, San Francisco). 4H84 is prepared as disclosed in Canadian Patent Application 2,213,620 (Fisher et al.), filed March 21, 1996. The mAb was generated by using a synthetic peptide corresponding to amino acids 61-83 of α1 domain of HLA-G (McMaster et al. J Immunol 1998: 60:5922-5928). The second, mAb 3C/G4, was produced by using

10   purified HLA-G protein to immunize BALB/c mice (Charles River), as described herein. Three week old female mice were given i.p. injections of 100 μg purified protein/animal and were boosted after 2 repeat injections. Mice were killed three days later and splenocytes were fused with SP2/0 myeloma cells according to standard methods, after which cultures were selected in hypoxanthine-ampinopterin-

15   thymidine medium and cloned by the limiting dilution method (Harlow E, Lane D: Monoclonal antibodies, *In: Antibodies: a Laboratory Manual*, edited by Harrow E and Lane D, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp 139-243, 1988).

Hybridomas were screened for reactivity against the purified HLA-G protein

20   by Ab-capture ELISA. Lines that produced anti-HLA-G antibodies were further screened for their ability to react with the HLA-G heavy chain in cytotrophoblast and JEG-3 cell lysates by western blot and immunocytochemistry. Pooled white blood cell lysates were used to select lines that do not react with classical class I antigens. Comparison of antibody binding sites were examined by using antibody

25   competition assay. 3C/G4 mAb was chosen because the mAb had the same specificity as 4H84 mAb, but it binds to a different epitope on HLA-G protein than 4H84 mAb . The class of the Ab was Ig A that was determined using an ImmunoType Kit (Amersham, Buckinghamshire, England).

The inventive ELISA was used for HLA-G analysis in serum and placenta lysates. The ELISA was developed based on the principles described by Micallef *et al.* (Micallef J, Ahsan R: Immunoassay development, In: *Immunoassay: Laboratory Analysis and Clinical Applications*, Eds by Gosling JP and Basso LV, pp51-68, Butterworth-Heinemann, London, 1994). Each well of a 96-well immunoplate

5   (Corning, NY, USA) was filled with 0.1 ml of 4H84 mAb (10µg/ml) and kept at 4 °C overnight. Each well was washed three times with a 0.01 M PBS washing solution containing 0.05% Tween-20 and blocked with 1% BSA for 4 hours at room temperature. Serum or placenta lysate samples were added to a final volume of 0.1 ml in each well and incubated at 4 °C overnight. Wells were washed three times

10 with the washing solution and to each well 0.1 ml of biotinylated 3C/G4 at optimum dilution was added. Plates were incubated for 2 hours at room temperature. All the wells were washed three times before adding 0.1 ml/well of 1:2000 dilution of streptavidin-HRP (Sigma Chemical Co., Louis, MD, USA) in 0.01M PBS containing 1% BSA and incubated for 1 hour at room temperature. Wells were

15 washed three times before adding o-phenylenediamine substrate solution (1.5 mg/ml in 0.1 M acetic buffer, pH=4.5). After 10-15 minutes incubation at room temperature the reactions were then stopped by the addition of 0.1 ml of 0.1N H_2SO_4 to each well and plate was read at 490 nm on an automated ELISA plate reader (Molecular Devices, USA). Curve fitting and dose interpolation were performed by

20 software associated with the reader. Total protein concentrations of placenta lysates were determined by the Bio-Rad™ protein assay kit (Bio-Rad, USA). For statistical analysis, differences of HLA-G levels between normal pregnancy and pre-eclampsia were assessed for statistical significance using Mann-Whitney rank sum test. Least squares linear regression was used to correlate HLA-G protein levels in serum and

25 placenta lysates.

   Results. ELISA validation was conducted. The immunoaffinity purified HLA-G protein was used as standard in ELISA.

As illustrated in Figure 8, the purity and characterization of purified HLA-G

protein, as analyzed by SDS-PAGE and immunoblot, conducted as described above.

The blot indicates that there are membrane bound and secreted HLA-G proteins.

These data illustrate that the purification of standard was estimated more than 95%.

The purified protein contained both the surface (38-39 Kd) and secreted forms (34-

5   35 Kd) of HLA-G. No classical class I molecules could be detected at molecular

weights in the 43 to 45 Kd range.

Figure 11 shows a typical standard curve by HLA-G ELISA. This standard

curve for the assay demonstrates a linear association between protein concentration

and OD at 490 nm. The assay limit was less than 10 ng/ml. Variations of within

10   and between assays were monitored using a placenta lysate pool of first trimester

placenta tissues and the coefficients of variations obtained was less than 10%.

The overall recovery of purified HLA-G spiked into serum and placenta

lysate was 101 ± 6.5 %, based on the data presented in Table 5 for added values

ranging from 0 to 0.8 μg. Serially diluted serum and placenta lysate gave dose

15   responses paralleled to that of the purified HLA-G protein further validating the

inventive ELISA assay.

Serum concentrations of HLA-G protein in maternal serum samples were

measured in 14 normal pregnant women. Values ranged from 0.016 to 4.396 with a

median of 0.093 mg/ml. In contrast, HLA-G concentrations measured in serum

20   from 20 patients with pre-eclampsia ranged from 0.009 to 0.590 with a mean of

0.026 mg/ml. The concentrations of serum HLA-G protein in pre-eclampsia were

significantly lower than that in normal pregnant women, as shown in Table 8.

30

Table 8

Serum and Placental HLA-G Levels in Normal Pregnant Women
and Women with Pre-Eclampsia at Term

	Normal Pregnant N=14	Pre-eclampsia N=20
	Serum HLA-G µg/ml	
Median	0.093*	0.026*
25%	0.039	0.016
75%	0.789	0.048
	Placental HLA-G µg/mg protein	
Median	0.088**	0.026**
25%	0.019	0.014
75%	0.124	0.056

*   Mann-Whitney Rank Sum Test: T=318, p=0.0112

**   Mann-Whitney Rank Sum Test: T=304, p=0.0406

Analysis of HLA-G protein in placental lysates from women with pre-eclampsia provided the following results. HLA-G protein concentrations in placenta lysate samples were normalized by their total protein concentrations representing as µg/mg protein. The HLA-G protein levels assayed in normal pregnant women ranged from 0.0065 to 0.2 with a median of 0.088 µg/mg protein. Patients with pre-eclampsia had concentrations ranging from 0.001 to 0.106 with a mean of 0.026 µg/mg protein. Consistent with the results in serum, placental HLA-G levels in normal pregnant women were significantly higher than those of pre-eclampsia patients.

Figure 12 illustrates the correlation of HLA-G protein concentrations in placenta and serum at term. HLA-G concentrations in normal pregnant women at term are represented by the closed circles (●), and the pre-eclampsia by open circles (O). The data were analyzed by linear regression analysis (r=0.603, p=0.0002). These results show that there was a significant correlation between serum HLA-G

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concentrations and HLA-G levels in placenta lysates from the same patients (r = 0.603, p = 0.0002).

Discussion. According to the invention, two monoclonal antibodies to HLA-G were used that bind to different epitopes on the molecule to develop a highly sensitive and specific ELISA for measurement of this molecule's concentration.

5   The invention demonstrates for the first time that the quantity of HLA-G protein in pre-eclamptic placental tissue is significantly decreased as compared to normal pregnant placentas. These results confirm the findings by Hara *et al.*, who used immunohistochemistry (Am. J. Reprod. Immunol 1996: 36:349-35), and Lim *et al.*, (Am. J. Pathol 1997;151: 1809-1818), who used Northern blot analysis to show

10   decrease HLA-G mRNA levels in isolated cytotrophoblast cells from pre-eclamptic placentas. Using ribonuclease protection assay, Globern *et al.* (Am.J.Obstet.Gynecol 1994:170: 1244-1250) reported that the level of HLA-G mRNA in pre-eclamptic placenta was reduced.

It is well established that extravillous cytotrophoblasts secrete a truncated

15   form of HLA-G, but there are no reports determining HLA-G concentrations in the serum of pregnant women. The invention demonstrates the presence of HLA-G in serum obtained from normal pregnant women and women with pre-eclampsia by using our HLA-G specific ELISA. Serum HLA-G concentrations in women with pre-eclampsia were significantly lower than that in normal pregnant women. In

20   addition, there was a significant correlation between serum HLA-G concentrations and placenta lysate levels, (as shown in Figure 13).

Although HLA-G mRNA has been detected in other adult and fetal tissues by RT-PCR analysis (Crisa *et al.*, J Exp.Med 1997: 186: 289-298), protein has not been detected in these tissues. Therefore, the placenta is the likely source of the

25   majority of, if not all of the HLA-G detected in serum. The restricted expression and minimally monomorphic nature of HLA-G have resulted in many to hypothesize that HLA-G could serve as "universally acceptable identity card " to allow trophoblast cells to evade rejection by the maternal immune system. How HLA-G might function in this capacity is becoming more unclear. It has previously been

demonstrated that HLA-G may prevent fetus-placenta unit from NK cell lysis. However, the instant inventors have demonstrated that HLA-G may inhibit the generation of cytotoxic cell activity (see Kupasi *et al.*, Immunology 2000:101:1-14).

It is unknown what HLA-G may play, if any, in the pathophysiology of pre-eclampsia. Pre-eclampsia has been designed as a two stage disease; the initial stage

5   involves poor placentation and the second stage is associated with placental ischemia. Clearly, immune mechanisms may have a primary role to play in the first stage of the pathogenesis of pre-eclampsia. In this stage, the impaired expression of HLA-G may not prevent the fetus-placenta unit from maternal immune rejection and subsequent fetal loss. With a milder degree of impairment, the pregnancy could

10   continue with later evolution to syndrome from chronic immune attack resulting in placental ischemia. Some evidence suggests that cytokines such as tumor necrosis factor (TNF-α) and interleukin (IL)-2 are increased in plasma and decidua from pre-eclamptic patients and serum IL-4 levels were decreased in pre-eclampsia. T cells that produce TNF-α, IL-2 and IFN-γ have been designated as Th1-type cells while T

15   cells that secrete IL-3, IL-4, IL-10, IL-13 and TGF-β2 are Th2-type cells. Evidence would suggest that a Th1-type response is detrimental whereas a Th2-type response is beneficial for pregnancy (Clark, *Am.J. Reprod. Immunol*, 1997: 38:75-78).

HLA-G in high concentrations of HLA-G result in a Th1-type response, whereas low concentrations of HLA-G result in a Th1-type response. Thus, the

20   decrease of HLA-G expression in pre-eclampsia may be associated with alternations in the maternal immune response pattern corresponding to a Th1 type.

In conclusion, the invention demonstrates that HLA-G levels in both serum and placenta were decreased in women with pre-eclampsia at term as compared to normal pregnancy. Serum levels are significantly correlated with placental levels in

25   these two groups. Alternations in HLA-G expression may play a role in pathophysiology of pre-eclampsia.

Example 3

Differences in Serum Human Leukocyte Antigen-G (HLA-G) in Normal and

Pre-eclamptic Women at During Different Trimesters of Pregnancy

Serum samples were collected from normal pregnant women or from pre-eclamptic women at first trimester, second trimester and third trimester (N=12). An

5 ELISA assay according to the invention was conducted, using 96-well plates prepared with immobilized 3C/G4 antibody, in which samples were incubated. HRP-labeled 2C/C8 binding was detected with OPD.

Figure 13 illustrates the data points and median values for HLA-G levels in serum taken from women in the first trimester of their pregnancy either during a

10 normal pregnancy, shown in closed circles (●), or during pre-eclampsia, shown by open circles (O).

Figure 14 illustrates the data points and median values for HLA-G levels in serum taken from women in the second trimester of their pregnancy either during a normal pregnancy, shown in closed circles (●), or during pre-eclampsia, shown by

15 open circles (O).

Figure 15 illustrates the data points and median values for HLA-G levels in serum taken from women in the third trimester of their pregnancy either during a normal pregnancy, shown in closed circles (●), or during pre-eclampsia, shown by open circles (O).

20 These data illustrate that the inventive assay is effective in the detection of serum HLA-G, and also that early stages in pregnancy (first and second trimester) are best for detection of pre-eclampsia based on serum HLA-G, as this is when differences between normals and pre-eclamptic subjects are most pronounced.

Example 4

25 *Progesterone Increases HLA-G Expression in vitro*

HLA-G is expressed on placental tissues at the maternal-fetal interface. This restricted pattern of expression suggests that HLA-G may play a crucial role in maternal immune tolerance to the fetus. It has been reported that HLA-G expression

is developmentally regulated and changes with stage of gestation. There are
conflicting reports that the cytokine gamma IFN has an effect on HLA-G expression
in vitro. Since, some studies suggest that progesterone is important in suppressing
the maternal immunological response to the fetus, it was hypothesized that this
steroid hormone may play a role in regulating HLA-G expression. The present study

5   was performed to explore potential effects of progesterone on HLA-G expression in
cytotrophoblasts and in the JEG-3 choriocarcinoma cell line *in vitro*.

Methods. Purified cytotrophoblasts were prepared from first trimester
placental tissue by standard techniques. JEG-3 choriocarcinoma cells were cultured
in RPMI-1640 medium containing 10% FCS for three days. The cells were then

10   treated with various doses of progesterone for 24 hours. Cell lysates and
conditioned media were prepared for ELISA and total RNA was extracted for PCR.
The ELISA was conducted according to the invention, using the 3C/G4 and 2C/C8
anti-HLA-G monoclonal antibodies. HLA-G protein, purified by immunoaffinity
columns from first trimester placental tissue, was used as standard. The sensitivity

15   of the ELISA was 20 ng/ml and assay variation was less than 10%. Quantitative
RT-PCR was performed by using specific primers for HLA-G. Results were
assessed for statistical significance using one-way analysis of variance.

Results. HLA-G protein in placenta cell culture was significantly increased
by progesterone at doses of 100 ng and 1000 ng/ml. Progesterone also had a

20   stimulating effect on HLA-G concentration in both JEG-3 cell lysate and
conditioned medium with a significant increase at doses from 10 ng/ml. The results
of RT-PCR indicated an increase of HLA-G mRNA levels after progesterone
treatment in both JEG-3 and cytotrophoblast cells. Cells treated with a 1000 ng/ml
concentration of progesterone showed significantly higher HLA-G mRNA levels

25   than that measured after exposure to 10 and 100 ng/ml concentrations.

Conclusion. These data suggest that progesterone regulates HLA-G
expression *in vitro*. Progesterone may thus be involved in regulation of placental
HLA-G expression. Since HLA-G may play an important role in maternal immune
tolerance to the fetus, it is possible that one mechanism of immunosupression by

30   progesterone during pregnancy is by a means of modulating HLA-G expression.

35

Further, this study indicates that the method of HLA-G detection according to the invention is effective for measuring HLA-G from *in vitro* cell lysates, including carcinoma cell lines.

Example 5

Secretion of Human Leukocyte Antigen-G (HLA-G) by Human Embryos is Associated with a Higher IVF Pregnancy Rate

5   It is shown herein that transfer of human embryos that secrete HLA-G results in a higher IVF pregnancy rate than transfer of embryos lacking HLA-G secretion. The combination of HLA-G detection and a higher embryo cleavage rate is an excellent predictor of IVF outcome.

10   Human leukocyte antigen G (HLA-G) is a non-classical class I antigen that may play an important role in maternal-fetal immune tolerance (Rouas-Freiss *et al.* Proc. Nat. Acad. Sci. USA, 1997; 94:11520-5). In pregnancy, HLA-G is expressed on the placental cytotrophoblast cells at the maternal-fetal interface (Le Bouteiller *et al.* Immunol Rev. 1999; 167:233-44.). HLA-G protein is detectable by

15   immunocytochemistry (ICC) in some human pre-implantation embryos (Jurisicova *et al.* Proc. Nat. Acad. Sci. USA. 1996; 93:161-5).

In placenta and some chroriocarcinoma cell lines, alternative splicing of the HLA-G gene results in six different transcriptional isoforms, four of which encode potential membrane-bound products, while two others encode soluble proteins. In

20   normal tissues one surface bound (G1) and one soluble form have been shown to be translated. The G1 form is most analogous to the classical class I cell surface molecules. In some tumors and tumor cell lines the other forms may exist (Paul *et al.* Proc. Natl. Acad. Sci. USA. 1998, 95:4510-5.). Soluble HLA-G (sHLA-G) has been detected in amniotic fluid, and in maternal plasma and in cell culture

25   supernatants from trophoblast-derived cell lines (Rebmann *et al.* Tissue Antigens 1999:53: 14-22; Fournel *et al.*, Am J Reprod Immunol 1999: 42:22-29). Sibling embryos from patients that became pregnant with *in vitro* fertilization (IVF) were significantly more likely to express HLA-G on their cell surface than embryos from

patients that did not conceive (Jurisicova *et al.*, Fertil Steril 1996; 65:997-1002).
Therefore, this study was designed to determine whether human embryos secrete
HLA-G and to analyze any associations between sHLA-G detection and embryo
development or IVF pregnancy outcome. One objective of this investigation was to
ascertain whether human embryos secrete soluble human leukocyte antigen G

5   (sHLA-G) and whether the secretion of sHLA-G is associated with embryo
development and IVF pregnancy outcome.

       Design. Three hundred and eighty six embryo culture conditioned media
samples were collected from 137 IVF cycles. HLA-G concentrations in the samples
were assayed by a specific and sensitive ELISA. Potential associations between

10   sHLA-G and pregnancy rate as well as other factors known to influence IVF
outcomes were analyzed.

       Patients and IVF. A total 137 of couples undergoing IVF at the Success
Through Assistant Reproductive Technology (S.T.A.R.T) IVF laboratory in
Toronto, Canada were involved in this study. Ovarian stimulation was carried out

15   using a standard protocol as previously described (Gonen *et al.*, Fertil Steril 1990:
53:282-7; Segal *et al.*, Human Reprod 1992; 7:1210-3). From these patients, a total
of 386 embryo cultured medium samples were collected. All of these were from
embryos cultured *in vitro* for entire 72 hours. All samples were from cases where
intracytoplasmic sperm injection (ICSI) was used for fertilization (Palermo *et al.*,

20   Lancet 1992; 340: 17). These embryos were in the same culture medium for the
entire time 72 hours. Fertilization was confirmed by observation of two pro-nuclei.
The number of embryos cultured per well ranged from 1 to 7 with an average of
2.90. The embryo quality was scored by grade and number of blastomeres
according to the Veek classification system. Embryos with the highest grade and

25   number of blastomeres were selected for transfer and the remaining viable embryos
were cryopreserved. Successful pregnancy was defined as a live birth. Spontaneous
abortion was defined as the presence of a sonographically detectable sac in the
uterus. Chemical pregnancies were not included in the pregnant group.

       Embryo Culture Conditioned Medium. Embryos were cultured in HTF

30   medium containing 10% human serum albumin (GIBCO/BRL, Gaithersburg, MD,

                                            37

USA). Culture took place in 0.4 ml of medium under 500 µl of paraffin oil in a cell culture incubator (5.5% CO_2 at 37 °C). After three days the embryos were selected for transfer and approximately 200 µl of the culture medium from each well was collected and stored at -80 °C until assay. As a control, culture medium without embryos was included and frozen in the same manner.

5   ***Purification of HLA-G Protein.*** Native HLA-G protein from first trimester placental tissue as described herein. Briefly, the placenta tissues were homogenized with a Brinkmann polytron homogenizer at 4 °C. The homogenized tissues were washed three times with 0.05 M Tris-HCl/saline pH=7.4 before solubilizing in 0.5% NP-40 in 0.05 M Tris-HCl/saline containing 2.5 mM EDTA and 200 mM PMSF at 4

10   °C overnight. The lysate was obtained by centrifugation at 39,000 g for 30 minutes at 4°C. HLA-G protein was then isolated by sequential 4H84 mAb (against HLA-G) and βMM1 mAb (against β2-microglobumin) affinity columns. Purity of isolated HLA-G protein was confirmed by Western blotting with the 4H84 mAb and Coomasie blue staining of the gel.

15   ***Monoclonal Antibodies.*** Two mAbs against HLA-G protein were used for development of our ELISA. The first was the mAb 4H84 which was a gift of S. Fisher and M. McMaster (University of California, San Francisco). This mAb was generated by using a synthetic peptide corresponding to amino acids 61-83 within the α1 domain of HLA-G (McMaster *et al.*, J Immunol 1995; 154:3771-8).

20   The second monoclonal antibody 3C/G4, was produced according to the invention using purified HLA-G protein preparation to immunize BALB/c mice (Charles River). Three-week-old female mice were given i.p. injections of 100 µg purified protein/animal and were boosted with 2 repeat injections. The mice were sacrificed three days later and their splenocytes were fused with SP2/0 myeloma

25   cells according to standard methods (Harlow E, Lane D: Monoclonal antibodies. In: Harrow E and Lane D, eds. Antibodies: a laboratory manual. NY: Cold Spring Harbor Laboratory, 1998:139-243). Cultures were selected in hypoxanthine-ampinopterin-thymidine medium and cloned by the limiting dilution method.

Hybridomas were screened for reactivity against the purified HLA-G protein by Ab-

capture ELISA according to standard methods. Cell lines that produced anti-HLA-G

Abs were further screened for their ability to react with the HLA-G heavy chain in

cytotrophoblast and JEG-3 choriocarcinoma cell lysates by western blot and

immunocytochemistry. Pooled white blood cell lysates were used to select lines that

5   do not react with classical class I antigens. Comparison of antibody binding sites

was examined by using an antibody competition assay. 3C/G4 mAb was chosen for

the ELISA because it has the same specificity for HLA-G as 4H84 mAb, but it binds

to a different epitope on the HLA-G heavy chain, thereby having a different binding

region.

10   **Figure 16** illustrates an antibody competition assay to compare antibody

binding sites of two monoclonal antibodies against HLA-G (4H84 and 3C/G4). In

the assay, a fixed amount of biotin labeled purified 4H84 antibody was added

together with various doses of unlabeled 4H84 antibody (control) or unlabeled

3C/G4 antibody to an ELISA plate coated with purified HLA-G protein. 3C/G4

15   binds to a different epitope on HLA-G than 4H84 since no binding competition

between these two antibodies (open circles).

        ELISA for HLA-G in Embryo Culture Medium. The ELISA was developed

based on the principles described by Micallef et al (Micallef J, Ahsan R:

Immunoassay development. In: Gosling JP and Basso LV, eds. Immunoassay:

20   Laboratory Analysis and Clinical Applications. London: Butterworth-Heinemann,

1994:51-68). Each well of a 96-well immunoplate (Corning, NY, USA) was filled

with 0.1 ml of 4H84 mAb (10μg/ml) and kept at 4 °C overnight. Each well was

washed three times with a 0.01 M PBS washing solution containing 0.05% Tween-

20 and blocked with 1% BSA for 4 hours at room temperature. Duplicate embryo

25   cultured medium samples were added to a final volume of 0.1 ml in each well and

incubated at 4 °C overnight. Wells were washed three times with the washing

solution and to each well 0.1 ml of biotinylated 3C/G4 at optimum dilution was

added. The plate was incubated for 2 hours at room temperature. All the wells were

washed three times before adding 0.1 ml/well of a 1:2000 dilution of streptavidin-

HRP (Sigma Chemical Co., St. Louis, MD, USA) in 0.01M PBS containing 1%

BSA, and then incubated for 1 hour at room temperature. Wells were then washed

three times before adding 150 µl of TMP substrate solution (Sigma Chemical

Co.St.Louis, MD, USA). After 10-15 minutes incubation at room temperature the

reactions were then stopped by the addition of 50µl of 1 M HCl to each well and

5   plate was read at 450 nm on an automated ELISA plate reader (Molecular Devices,

USA). Curve fitting and dose interpolation were performed using software

associated with the reader.

Statistical Analysis. Embryo conditioned medium was scored as HLA-G

positive or negative according to whether sHLA-G was detectable or not by Elisa.

10   For the propose of these analyses, any cases where at least 1 embryo transferred

came from a HLA-G positive well was scored as positive. Differences concerning

patient's age, embryo grade, embryo cleavage rate, and number of embryos per

culture well between the two groups were assessed for statistical significance by

using the Student's t test or Mann-Whitney rank sum test depending upon whether

15   the data had a normal distribution. sHLA-G concentration and embryo cleavage rate

the in data was compared by using the Kruskal-Wallis one-way analysis of variance

on rank. Chi-square analysis was used to compare pregnancy rates between various

groups.

Results. 270 of 386 samples had detectable sHLA-G. Detection of sHLA-G

20   secretion was independent of embryo grade and patient's age. The cleavage rate of

embryos with detectable sHLA-G was significantly higher than those without

sHLA-G (number of blastomerers 6.71 ± 0.09 vs 5.86 ± 0.22, p = 0.0061). Live

birth rates from transferred embryos with positive sHLA-G secretion were

significantly higher than those with undetectable HLA-G (45% vs 16.2%, $\chi 2=8.38$,

25   p=0.0038). Combining sHLA-G and cleavage rate were the best prediction of IVF

success. Secreted HLA-G (sHLA-G) was detectable in 270 out of 386 embryo conditioned medium samples (69.9%) and concentrations ranged from 0.01 μg/ml to 1.89 μg/ml with a mean of 0.165 μg/ml. Age, embryo grade and number of embryos per culture well were compared between HLA-G detectable and undetectable groups.

5   Table 9 illustrates that there were no significant differences in these factors between the two groups. Samples were grouped into HLA-G negative and positive groups according to whether sHLA-G was detectable by the ELISA or not.

Table 9

Comparison of Patient's Age, Embryo Grade and Number of Embryos per Culture
10   Well Between HLA-G Negative and Positive Groups

	Patient's Age (yr)**	Grade of embryos*	No. Embryos/well*
HLA-G (-), N=116	35.0 ± 0.52	2.40 ± 0.08	2.80 ± 0.14
HLA-G(+), N=270	34.4 ± 0.29	2.41 ± 0.04	2.83 ± 0.08
Significance	NS	NS	NS

*Tested by Mann-Whitney rank sum test. ** Tested by Student's t test. All data
15   are presented as mean ± SE.

Figure 17 shows that a significant difference in embryo cleavage rate (number of blastomeres: 6.76 ± 0.09 vs 5.86 ± 0.22, p=0.0061) between the two groups was observed. The HLA-G positive group samples were further divided into four sub-groups according to average number of blastomeres per culture well (group
20   1: ≤4, group 2: >4=6, group 3:>6=8 and group 4: >8) and average HLA-G concentrations were compared. Samples were grouped according to whether medium sHLA-G was detectable or not. Using Student's t test compared average number of blastomeres per culture well between sHLA-G positive and negative

groups. (*)Indicates a significant difference between two groups (t = -2.77, p=0.0061).

Table 10 illustrates the comparison between HLA-G secretion by human embryos and embryo clevage rate.

Table 10

5   Association Between HLA-G Secretion by Human Embryos and Embryo

Cleavage Rate

	Number of Blastomeres			
	≤4 (N=56)	>4-6 (N=86)	>6-8(N=116)	>8(N=12)
	HLA-G (µg/ml)			
Median	0.104	0.190	0.180	0.308
25-75%	0.049-0.243	0.075-0.373	0.115-0.376	0.149-0.343

10   Samples were grouped according to average number of blastomeres per embryo in each well. Association between sHLA-G concentrations and embryo cleavage rate was analyzed by using Kruskal-Wallis one way analysis of variance on rank. H=15.1 and p=0.0018. It was found that sHLA-G concentrations were significantly different between these groups (Kruskal-Wallis, p=0.0018). sHLA-G

15   concentration was lowest with embryos less than 4 blastomeres and greatest with >8 blastomeres.

Of the total 137 couples, 51 had a live birth, 9 had a spontaneous abortion and 77 did not achieve a pregnancy. Similar to findings from other centers (Ziebe *et al.*, Human reprod 1997; 12:1545-9), the pregnancy rate from embryos with average

20   > 6 of cells was tended to be higher than that from embryos with ≤ 6 cells (40.9% vs 30.6%), but this did not reach statistical significance in our study. For this difference to be significant with a *p* value of 0.800, a total of 440 subjects would have been required.

Figure 18 illustrates the pregnancy rate in patients who had sHLA-G

positive embryos transferred was significantly higher than that in patients who

received sHLA-G negative embryos (45% vs 16.2%, Chi-square =8.38, p= 0.0038).

Pregnancy rates were compared between HLA-G positive and negative groups and

between average number of blastomeres larger than 6 and less/equal to 6 groups by

5   using Chi-square testing. A significant difference between two HLA-G groups was

observed, while no statistical significance was not found between the cell number

groups.

HLA-G positivity was a better independent predictor than cleavage rate for

successful pregnancy. Of all pregnancy cases 88.8% resulted from transfer of

10   embryos that were HLA-G positive. Only 16.2% of embryos transferred from HLA-

G negative wells gave rise to a pregnancy. To determine if prior knowledge of

HLA-G detection could have potentially improved pregnancy rates, those cases in

which the investigators unknowingly transferred HLA-G negative embryos and

froze HLA-G positive embryos were analysed. In total there were 17 cases where

15   this occurred. Only 2/17=11.8% of these cases resulted in a pregnancy.

In order to combine sHLA-G detectability and cleavage rate, all transferred

embryos were subdivided into four groups: group I were sHLA-G negative

combined with a low cleavage rate (average blastomere $\leq$ 6); group II were sHLA-G

negative combined with a high cleavage rate (average blastomeres >6); group III

20   were sHLA-G positive combined with a low cleavage rate and group IV were sHLA-

G positive combined with a high cleavage rate.

Figure 19 illustrates that differences in pregnancy rate among these groups

were statistically significant. The lowest pregnancy rate was seen in the combined

HLA-G negative and low cleavage rate group (7.7%) and the highest pregnancy rate

25   was observed in the combined sHLA-G positive and high cleavage rate group

(48.4%) (Chi-square = 11.1, p=0.0112). Pregnancy rates were compared among

various groups combining sHLA-G detection and embryo cleavage rate. Group I:

combined sHLA-G =0 and number of blastomeres $\leq$ 6; group II: sHLA-G =0,

number of blastomeres>6; group III: HLA-G >0, number of blastomeres $\leq$6; and

30   group IV: sHLA-G >0, number of blastomeres >6 (Chi-square = 11.1, p=0.0112).

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3C/G4 was shown to be an IgA mAb as determined using an ImmunoType kit (Amersham, Buckinghamshire, England).

Table 11 shows results obtained for patients according to age. As expected, pregnancy rates were higher in women < 38 years old as compared to those ≥ 38 years old. Pregnancy rates were significantly higher in either group if HLA-G was

5 detected in medium from transferred embryos.

Table 11

Comparison of Pregnancy Rates by Age and sHLA-G Detection.

	<38 years old				≥ 38 years old			
	HLA-G (+)		HLA-G (-)		HLA-G (+)		HLA-G (-)	
	N=74		N=28		N=26		N=9	
	N	%	N	%	N	%	N	%
Total Pregnant	43	58.1	6	21.5	11	42.3	0	0
Spontaneous Abortion	6	8.1	0	0	3	11.5	0	0
Live Birth	37	50.0	6	21.5	8	30.8	0	0

Chi-square = 20.7, p = 0.0021.

Patients were grouped according to their age < 38 or ≥ 38 years. Pregnancy rate was compared among subgroups according to whether HLA-G positive embryos

15 were transferred or not by Chi-square analysis.

Discussion and Conclusions. Embryo secretion of sHLA-G protein is variable. Secretion of HLA-G is correlated with a higher embryo cleavage rate. Embryos secreting soluble HLA-G have a higher IVF pregnancy rate than those lacking HLA-G secretion and detection of sHLA-G was a better prediction of

20 pregnancy outcome than cleavage rate alone. Combined sHLA-G detection and high cleavage rate was the best predictor of IVF outcome.

In vitro fertilization has become one of the most widely used methods for the treatment of human infertility. At present, two main determinants are used for selection of embryos for transfer. Firstly, embryo morphology grading is widely used. Various grading systems by different laboratories are utilized that take into account such factors as cell shape, cell size disparity, fragment percentage, and

5   fragmentation pattern. Secondly, cleavage status before transfer is determined by counting the number of blastomeres present. Both factors have some utility, but are relatively poor predictors of subsequent implantation and pregnancy. Average embryo grade was not predictive of pregnancy in our study agreeing with many other reports (Grimbizis *et al.* Human Reprod 1998; 13: 884-9). Pregnancy rate

10   tended to be higher with a higher average cleavage rate, but this did not reach statistical significance.

Using the specific and sensitive ELISA according to the invention, this study demonstrated that some pre-implantation embryos secrete HLA-G. This finding supports previous uses of ICC to detect membrane bound and intracellular HLA-G

15   (Jurisicova *et al.*, *Proc. Nat. Acad. Sci. USA.* 1996; 93:161-5). The soluble HLA-G detected by the ELISA according to the invention could be either the form in which a post transcriptional mRNA modification results in deletion of the transmembrane portion of HLA-G, or by proteolytic cleavage of a membrane bound form, or both. In this study, we found no association between sHLA-G detection and embryo

20   morphology.

A positive correlation was found between sHLA-G concentrations and embryo cleavage rate. Since it has been reported that the Ped gene and its product the Qa-2 antigen influence the cleavage rate of mouse embryos, HLA-G could be a functional homologue of the Qa-2 antigen, proposed by Cao *et al.* (*Mol Human*

25   *Reprod.* 1999; 5: 541-7). Therefore, sHLA-G, which is secreted by embryos could enhance embryo development. This could also be an explanation for why cleavage rate is improved when embryos are cultured in groups.

A positive association exists between HLA-G expression in sibling embryos and implantation after IVF. In this study, sHLA-G was measured in culture medium

30   from embryos before uterine transfer. This provided a means for directly detecting

45

any association between HLA-G produced by the embryos actually transferred and pregnancy outcome. In this study we demonstrated that transferred embryos which secrete detectable levels of HLA-G give rise to a significantly higher pregnancy rate than those which were HLA-G negative. The combination of a positive sHLA-G test and a higher cleavage rate gave rise to the highest pregnancy rate. Therefore,

5   advantageously, these two tests combined could be used to select embryos more likely to result in a pregnancy. The data was further analysed to see if there were cases in which this test alone would have changed the decision of which embryos to transfer. There were 2 pregnancies out of 17 cases (11.8%) in which the embryos transferred were sHLA-G negative and the spare embryos that were frozen were

10   HLA-G positive. Therefore, transferring the sHLA-G positive embryos in this group may have resulted in a higher pregnancy rate.

In addition to the potential role of HLA-G in improving embryo cleavage rate, as discussed above, sHLA-G may also play a role in communication with the maternal immune system to prevent rejection of transferred embryos. This

15   hypothesis is supported by the findings that higher pregnancy rates resulted from embryos with detectable sHLA-G even when the cleavage rate was low and by observations that purified HLA-G prevented the generation of an allo-response *in vitro*.

In summary, this study demonstrates variable detection of sHLA-G in human

20   embryo culture conditioned medium by the ELISA for HLA-G according to the invention. A significantly higher pregnancy rate is observed when embryos that secrete sHLA-G are transferred. Secretion of HLA-G was a better independent predictor of pregnancy rate than cleavage status alone. Finally, combining sHLA-G detection and cleavage rate was the best overall predictor of IVF pregnancy

25   outcome.

Example 6

Preparation of a Miniaturized HLA-G Detection Assay

As shown in Example 5, pregnancy outcomes from transferred embryos

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testing positive for sHLA-G secretion are significantly better than those with undetectable sHLA-G. Thus, to ascertain whether an embryo to be transferred has an increased likelihood of leading to pregnancy, an HLA-G detection assay can be conducted. The ELISA according to the invention is adapted to a miniaturized HLA-G detection assay which advantageously can be used in a clinical setting.

5   Such a miniaturized assay provides a rapid quantitative measurement of HLA-G secreted into a medium in which an embryo is incubated, without requiring that a sample be sent off for laboratory analysis.

A polystyrene chip is prepared by coating with 3C/G4 antibody according to the invention to form an active region. A 10 µl sample of embryo incubation

10   medium is placed on the active region of the chip. HLA-G is detected using HRP-2C/C8 conjugate, according to the invention, by inserting the chip containing the sample into a hand-held reader, as disclosed in International Patent Application No. PCT/CA99/01079, to Peter Lea,

published as International Publication No. WO 00/29847 on May 25, 2000. This

15   document is incorporated herein by reference in its entirety.

A fluorescent end-point is used to detect binding of antibodies, indicative of the presence of HLA-G. Advantageously, in an optional embodiment, the chip allows separation of fluid from non-fluid components of a sample. The device is a point-of-care device, which may be used in a clinical setting. The data is

20   electronically collected and/or transmitted for further evaluation.

Example 7

Preparation of a Cancer Screening Kit

HLA-G secretion from various cell types may be indicative of cancer. Certain cancerous states, such as breast cancer, can be screened using a kit according

25   to the invention. Thus, to ascertain whether a tissue sample may be cancerous or has potential to become cancerous, an HLA-G detection assay can be conducted. The appropriate tissue sample is collected, in this case from a breast tissue biopsy. Cells from the biopsy may be used as primary culture, or the sample may be homogenized

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according to methods disclosed herein with reference to placental tissue.

An ELISA is prepared by coating a support with 3C/G4 antibody. A 100 µl sample of homogenized tissue is placed on the coated support. HLA-G is allowed time to bind to the support, washed, and an aliquot of a HRP-2C/C8 conjugate is placed thereon. The conjugate binds to HLA-G remaining on the support. A non-

5 cancerous standard, such as a known value, or a value previously determined in the same individual from a previous non-cancerous biopsy is used to compare with the sample value of HLA-G. Threshold values of HLA-G indicative of cancerous and non-cancerous states are used for comparison with values measured in the tissue sample.

10 All references noted herein are incorporated by reference. The foregoing examples are merely indicative of embodiments of the invention, and should in no way be construed as limiting.

Deposit Receipt 2C/C8

International Depositary Authority of Canada
Bureau of Microbiology, Health Canada
Room H5190, 1015 Arlington Street,
Winnipeg, Manitoba, Canada R3E 3R2

Tel: (204) 789-2002
Fax: (204) 789-2036

International Form IDAC/BP/4

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
(issued pursuant to Rule 7.1 of the *Budapest Treaty* Regulations)

ATTACH COPIES OF THE ORIGINAL DEPOSIT CONTRACT AND VIABILITY STATEMENT

This International Depository Authority accepts the deposit of the microorganism specified below, which was received by it on _____ Sep 13, 2000 _____

To (Name of Depositor) _____ Clifford L. Librach _____

Address _____ 790 Bay Street, Suite 1020 _____
_____ Toronto, ON, M5G 1N8 _____

IDENTIFICATION OF DEPOSIT

Reference assigned by depositor _____ Hybridoma 2C/C8 _____

Accession Number assigned by this IDA _____ IDAC 130900-1 _____

The deposit identified above was accompanied by:

☐   a scientific description (specify) _____

_____

☐   a proposed taxonomic designation (specify) _____

_____

Signature of person(s) authorized to represent IDAC:

Date _____ Sep 13, 2000 _____

Deposit Receipt 3C/G4

International Depositary Authority of Canada
Bureau of Microbiology, Health Canada
Room H5190, 1015 Arlington Street,
Winnipeg, Manitoba, Canada R3E 3R2

Tel: (204) 789-2002
Fax: (204) 789-2036

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To (Name of Depositor) _____ Clifford L. Librach _____

Address __ 790 Bay Street, Suite 1020 _____
             Toronto, ON, M5G 1N8

IDENTIFICATION OF DEPOSIT

Reference assigned by depositor ____ Hybridoma 3C/G4 _____

Accession Number assigned by this IDA ___ IDAC 130900-2 _____

The deposit identified above was accompanied by:

☐   a scientific description (specify) _____

☐   a proposed taxonomic designation (specify) _____

Signature of person(s) authorized to represent IDAC:

Date ____ Sep 13, 2000 _____

Indications relating to IDAC 130900-1

Applicant's or agent's file reference number	PAT 1982W-90	International application No.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description

on page _____ **96** _____ , line _____ **5 9** _____ .

B. IDENTIFICATION OF DEPOSIT   Further deposits are identified on an additional sheet ☒

Name of depositary institution

International Depositary Authority of Canada (IDAC)

Address of depositary institution *(including postal code and country)*

Bureau of Microbiology, Health Canada
Room H5190, 1015 Arlington Street,
Winnipeg, Manitoba, CANADA R3E 3R2

Date of deposit	Accession Number
September 13, 2000	IDAC 130900-1

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)*   This information is continued on an additional sheet ☐

The applicant hereby requests that until either a patent has been issued on the basis of this application or the application is refused, or is abandoned and no longer subject to reinstatement, or is withdrawn, the Commissioner of Patents for each designated State, including Canada and the United States, only authorize the furnishing of a sample of the deposited biological material to an independent expert nominated by the Commissioner. Under such circumstances and within a reasonable time period, the Commissioner is requested to nominate a person as an independent expert for the purposes of this application, with agreement of the applicant.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g. "Accession Number of Deposit")*

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Authorized officer	Authorized officer
Carole Millein	

Form PCT/RO/134 (July 1992)

Indications relating to IDAC 130900-2

Applicant's or agent's file reference number	PAT 1982W-90	International application No.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description

on page _____ 56 _____ . line _____ 14 _____ .

B. IDENTIFICATION OF DEPOSIT   Further deposits are identified on an additional sheet ☒

Name of depositary institution

     International Depositary Authority of Canada (IDAC)

Address of depositary institution *(including postal code and country)*

Bureau of Microbiology, Health Canada
Room H5190, 1015 Arlington Street,
Winnipeg, Manitoba, CANADA R3E 3R2

Date of deposit	Accession Number
September 13, 2000	IDAC 130900-2

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)*   This information is continued on an additional sheet ☐

The applicant hereby requests that until either a patent has been issued on the basis of this application or the application is refused, or is abandoned and no longer subject to reinstatement, or is withdrawn, the Commissioner of Patents for each designated State, including Canada and the United States, only authorize the furnishing of a sample of the deposited biological material to an independent expert nominated by the Commissioner. Under such circumstances and within a reasonable time period, the Commissioner is requested to nominate a person as an independent expert for the purposes of this application, with agreement of the applicant.

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CLAIMS:

1.　　A method for detecting HLA-G in a biological sample comprising the steps of:

a) depositing a biological sample on a support having an immobilized anti-
HLA-G antibody bound thereto; said immobilized anti-HLA-G antibody binding to a first region of HLA-G;

b) contacting the support having the biological sample deposited thereon with an HLA-G label, said label binding to a second region of HLA-G; and

c) detecting said label.

2.　　The method according to claim 1, wherein said immobilized anti-HLA-G antibody is selected from the group consisting of 2C/C8 from 2C/C8 hybridoma deposited as IDAC Accession Number 130900-1 on September 13, 2000; 3C/G4 from 3C/G4 hybridoma deposited as IDAC Accession Number 130900-2 on September 13, 2000; and 4H84.

3.　　The method according to claim1, wherein said HLA-G label comprises a mobile anti-HLA-G antibody having a reporter molecule bound thereto.

4.　　The method according to claim 3, wherein:

said immobilized anti-HLA-G antibody is selected from the group consisting of 2C/C8 from 2C/C8 hybridoma deposited as IDAC Accession Number 130900-1 on September 13, 2000; 3C/G4 from 3C/G4 hybridoma deposited as IDAC Accession Number 130900-2 on September 13, 2000; and 4H84;

said mobile anti-HLA-G antibody is selected from the group consisting of 2C/C8, 3C/G4, and 4H84; and

provided that one of said immobilized anti-HLA-G antibody and said mobile anti-HLA-G antibody is 3C/G4.

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5.   The method according to claim 4, wherein the immobilized anti-HLA-G
antibody is selected from 2C/C8 and 4H84, and the mobile anti-HLA-G antibody is
3C/G4.

6.   The method according to claim 4, wherein the immobilized anti-HLA-G
antibody is 3C/G4 and the mobile anti-HLA-G antibody is selected from 2C/C8 and
5   4H84.

7.   The method according to claim 3, wherein said reporter molecule is selected
from the group consisting of colorimetric agents, fluorometric agents, radioisotopic
agents and enzymatic agents having a detectable end-point.

8.   The method according to claim 1, further comprising the step of measuring
10   HLA-G by comparing label detected in the biological sample with an HLA-G
standard.

9.   The method according to claim 8, wherein the HLA-G standard comprises
HLA-G from a human placenta.

10.   The method according to claim 1, wherein the biological sample is selected
15   from the group consisting of amniotic fluid, a medium contacting an embryo, a
tissue sample, a blood sample, a medium contacting a tissue sample, and a medium
contacting a cell.

11.   A method for identifying an HLA-G indicative condition in a patient
comprising the steps of:
20   a) obtaining a biological sample from the patient;
            b) depositing the biological sample on a support having an immobilized anti-
HLA-G antibody bound thereto; said immobilized anti-HLA-G antibody binding to

a first region of HLA-G;

     c) contacting the support having the biological sample deposited thereon with an HLA-G label, said label binding to a second region of HLA-G; and

     d) detecting said label.

12.   The method according to claim 11, wherein said immobilized anti-HLA-G antibody is selected from the group consisting of 2C/C8 from 2C/C8 hybridoma deposited as IDAC Accession Number 130900-1 on September 13, 2000; 3C/G4 from 3C/G4 hybridoma deposited as IDAC Accession Number 130900-2 on September 13, 2000; and 4H84.

13.   The method according to claim 11, wherein said HLA-G label comprises a mobile anti-HLA-G antibody having a reporter molecule bound thereto.

14.   The method according to claim 13, wherein:

     said immobilized anti-HLA-G antibody is selected from the group consisting of 2C/C8 from 2C/C8 hybridoma deposited as IDAC Accession Number 130900-1 on September 13, 2000; 3C/G4 from 3C/G4 hybridoma deposited as IDAC Accession Number 130900-2 on September 13, 2000; and 4H84;

     said mobile anti-HLA-G antibody is selected from the group consisting of 2C/C8, 3C/G4, and 4H84; and

     provided that one of said immobilized anti-HLA-G antibody and said mobile anti-HLA-G antibody is 3C/G4.

15.   The method according to claim 11, further comprising the step of comparing a detected level of said label with a control value for said HLA-G indicative condition.

16.   The method of claim 11, wherein said HLA-G indicative condition is

selected from pre-eclampsia, increased risk of pre-eclampsia, adverse fetal outcome, increased risk of adverse fetal outcome, cancer, and increased risk of cancer development.

17.   A method for determining potential for successful implantation of an embryo comprising the steps of:

5   a) obtaining a sample of a fluid medium incubating said embryo;

           b) depositing the sample on a support having an immobilized anti-HLA-G antibody bound thereto; said immobilized anti-HLA-G antibody binding to a first region of HLA-G;

           c) contacting the support having the sample deposited thereon with an HLA-

10   G label, said label binding to a second region of HLA-G; and

           d) detecting said label.

18.   The method according to claim 17, wherein said immobilized anti-HLA-G antibody is selected from the group consisting of 2C/C8 from 2C/C8 hybridoma deposited as IDAC Accession Number 130900-1 on September 13, 2000; 3C/G4

15   from 3C/G4 hybridoma deposited as IDAC Accession Number 130900-2 on September 13, 2000; and 4H84.

19.   The method according to claim17, wherein said HLA-G label comprises a mobile anti-HLA-G antibody having a reporter molecule bound thereto.

20.   The method according to claim 19, wherein:

20   said immobilized anti-HLA-G antibody is selected from the group consisting of 2C/C8 from 2C/C8 hybridoma deposited as IDAC Accession Number 130900-1 on September 13, 2000; 3C/G4 from 3C/G4 hybridoma deposited as IDAC Accession Number 130900-2 on September 13, 2000; and 4H84;

           said mobile anti-HLA-G antibody is selected from the group consisting of

2C/C8, 3C/G4, and 4H84; and

provided that one of said immobilized anti-HLA-G antibody and said mobile anti-HLA-G antibody is 3C/G4.

21.   Hybridoma 2C/C8 deposited at the International Depositary Authority of Canada on September 13, 2000 having IDAC Accession Number IDAC 130900-1.

5   22.   An anti-HLA-G antibody from the hybridoma of claim 21.

23.   An ELISA comprising the anti-HLA-G antibody of claim 22.

24.   An ELISA according to claim 23, comprising a miniaturized assay conducted on a chip.

25.   Hybridoma 3C/G4 deposited at the International Depositary Authority of
10   Canada on September 13, 2000 having IDAC Accession Number IDAC 130900-2.

26.   An anti-HLA-G antibody from the hybridoma of claim 25.

27.   An ELISA comprising the anti-HLA-G antibody of claim 26.

28.   An ELISA according to claim 27 comprising a miniaturized assay conducted on a chip.

15   29.   A kit comprising:
a) a support having an immobilized anti-HLA-G antibody bound thereto; said immobilized anti-HLA-G antibody binding to a first region of HLA-G;
b) an HLA-G label, said label binding to a second region of HLA-G.

30.   The kit according to claim 29, wherein said immobilized anti-HLA-G
antibody is selected from the group consisting of 2C/C8 from 2C/C8 hybridoma
deposited as IDAC Accession Number 130900-1 on September 13, 2000; 3C/G4
from 3C/G4 hybridoma deposited as IDAC Accession Number 130900-2 on
September 13, 2000; and 4H84.

5   31.   The kit according to claim 29, wherein said HLA-G label comprises a mobile
anti-HLA-G antibody having a reporter molecule bound thereto.

32.   The kit according to claim 29 for determining potential for successful
implantation of an embryo.

33.   A method for selecting an embryo for *in vitro* fertilization (IVF) comprising
10   the steps of detecting soluble HLA-G secreted by an embryo into an incubation
medium, and choosing an embryo secreting soluble HLA-G at a minimum level of
0.01 µg/ml of incubation medium.

34.   The method of claim 33, wherein embryo cleavage rate is evaluated and is
considered in combination with soluble HLA-G level in the step of choosing an
15   embryo.

35.   The method according to claim 33, wherein said step of detecting soluble
HLA-G comprises conducting the method of claim 1.

FIG. 1

FIG. 2

1) JEG-3
2) WBC
3) LCL
4) C1R

FIG. 3A
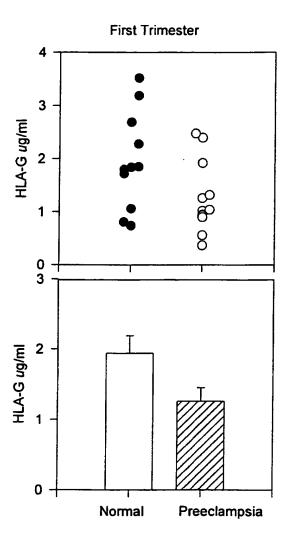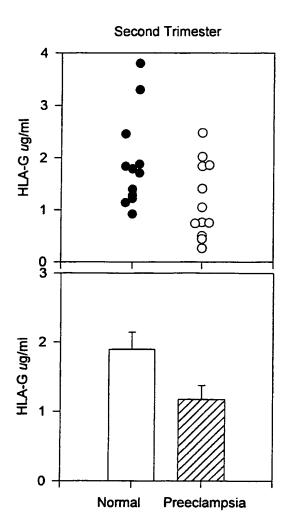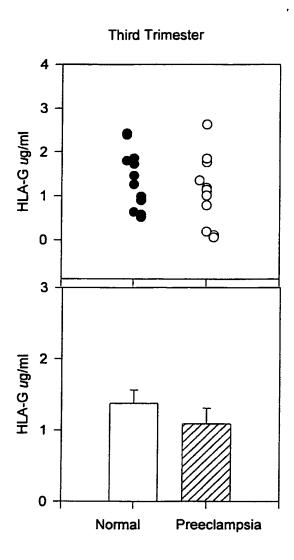
FIG. 3B

FIG. 4

FIG. 5

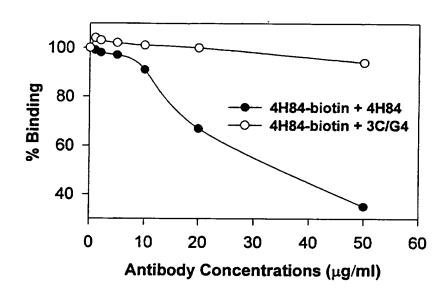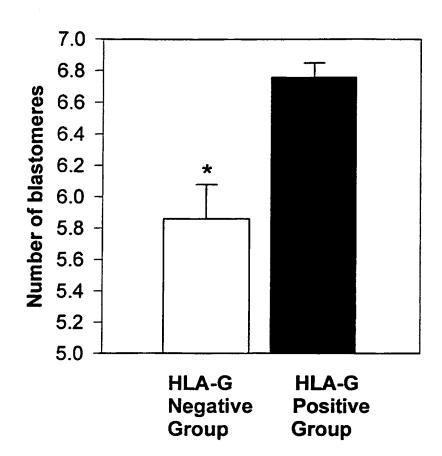FIG. 6
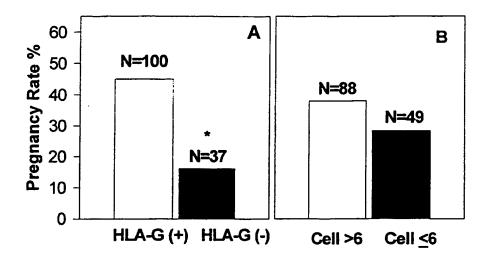
FIG. 7
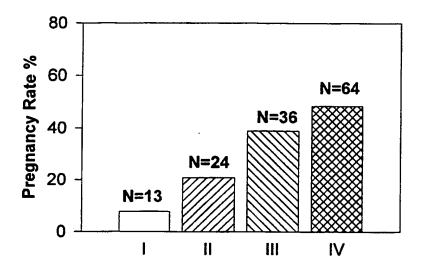
FIG. 8

FIG. 9

FIG. 10

FIG. 11

FIG. 12

FIG. 13

FIG. 14

FIG. 15

FIG. 16

FIG. 17

FIG. 18

FIG. 19